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Review

Supramolecular organization of thylakoid membrane proteins in green plants

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Abstract

The light reactions of photosynthesis in green plants are mediated by four large protein complexes, embedded in the thylakoid membrane of the chloroplast. Photosystem I (PSI) and Photosystem II (PSII) are both organized into large supercomplexes with variable amounts of membrane-bound peripheral antenna complexes. PSI consists of a monomeric core complex with single copies of four different LHCI proteins and has binding sites for additional LHCI and/or LHCII complexes. PSII supercomplexes are dimeric and contain usually two to four copies of trimeric LHCII complexes. These supercomplexes have a further tendency to associate into megacomplexes or into crystalline domains, of which several types have been characterized. Together with the specific lipid composition, the structural features of the main protein complexes of the thylakoid membranes form the main trigger for the segregation of PSII and LHCII from PSI and ATPase into stacked grana membranes. We suggest that the margins, the strongly folded regions of the membranes that connect the grana, are essentially protein-free, and that protein–protein interactions in the lumen also determine the shape of the grana. We also discuss which mechanisms determine the stacking of the thylakoid membranes and how the supramolecular organization of the pigment–protein complexes in the thylakoid membrane and their flexibility may play roles in various regulatory mechanisms of green plant photosynthesis.

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Keywords: Photosynthesis; Photosystem I; Photosystem II; Light-harvesting complex; Cytochrome *b₆/f* complex; Thylakoid membrane; Supercomplex; Grana; Cyclic electron transport; State transition; Nonphotochemical quenching

1. Introduction

The process of photosynthesis cannot be understood without a detailed knowledge of the structure of its single components. Over the last two decades a substantial effort

has been put on solving high-resolution structures of individual components, such as PSI [1,2], PSII [3–5], LHCII [6,7], ATPase [8,9] and the cytochrome *b₆/f* complex [10,11], and for all these complexes intermediate (3.5–4.2 Å) or high (<2.5 Å) resolution structures are available. There is also an increasing emphasis on the interaction of these complexes into higher order associates, like supercomplexes. This is not unique for the chloroplast, since also in the (plant) mitochondrion the existence of supercomplexes has been described, such as the dimeric form of the ATP synthase [12].

Within each chloroplast, the photosynthetic thylakoid membranes form a physically continuous three-dimensional network that encloses a single aqueous space, the thylakoid lumen. A characteristic feature of this mem-

Abbreviations: α-DM, *n*-dodecyl-α,D-maltoside; AFM, atomic force microscopy; C, monomeric photosystem II core complex; Chl, chlorophyll; EM, electron microscopy; FNR, ferredoxin-NADP-reductase; Isi, iron-stress inducible; L, loosely bound trimeric LHCII; LHCI, light-harvesting complex I; LHCII, light-harvesting complex II; M, moderately bound trimeric LHCII; Pcb, prochlorophyte chlorophyll a/b; PSI, photosystem I; PSII, photosystem II; qE, high-energy state quenching; S, strongly bound trimeric LHCII; VDE, violaxanthin de-epoxidase

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brane is its extensive folding (Fig. 1). As a consequence, the thylakoid membranes of vascular plants and some green algae are structurally inhomogeneous. They consist of two main domains: the grana, which are stacks of thylakoids, and the stroma lamellae, which are unstacked thylakoids and connect the grana stacks. Three-dimensional models of the spatial relationship between grana and stroma thylakoids show that PSII and LHCII reside mainly in the grana membranes, while PSI and ATPase reside predominantly in the stroma and the cytochrome *b₆f* complex is distributed about evenly between the two types of membranes.

In contrast to the many reviews on photosynthesis dealing with the subunit composition and structure of specific single complexes or with their mechanism, this review primarily focuses on the overall composition and structure of supercomplexes of green plant thylakoid membranes, their organisation and interactions in the photosynthetic membrane, and how the supramolecular organization influences the grana–stroma division and can play roles in the various photosynthetic regulation mechanisms.

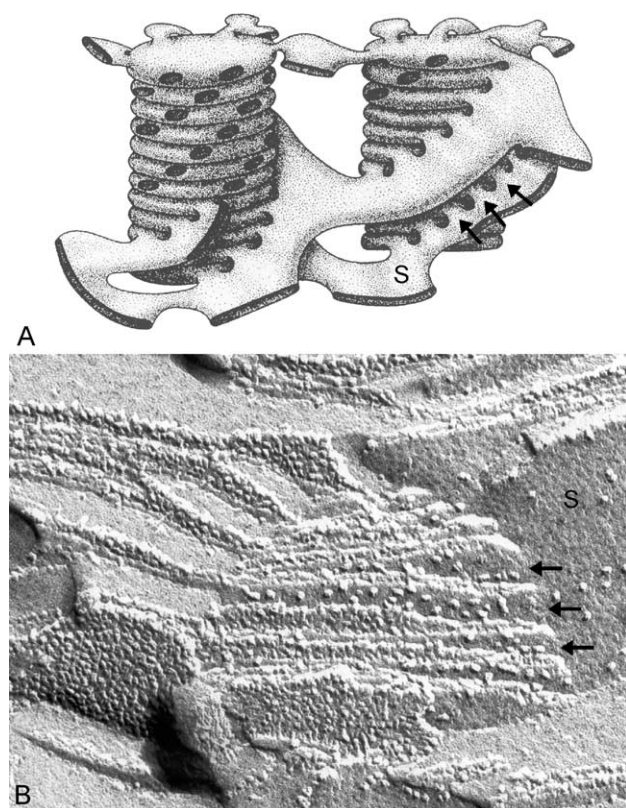


Fig. 1. (A) 3D model for the stacking of grana membranes. (B) Freeze-fracture electron microscopy showing the connections between a stroma membrane (S) and the grana stack (arrows; from Ref. [13], reprinted with permission). The model in (A) is merely based on freeze-fracture data. However, there is one constraint: the continuous membrane should rapidly make such grana stacks upon restacking, see Ref. [235]. This could mean that the position of the spikes is different from the model in (A) because this model does not clearly show how folding could occur.

2. Molecular organization of photosystem I, ATPase and cytochrome *b₆f*

2.1. PSI core complex

The structure of the PSI core complex from the cyanobacterium *Thermosynechococcus elongatus* is known at 2.5-Å resolution [1,14,15]. The structure of the PSI core complex is evolutionarily related to that of the PSII core complex (see, e.g., Refs. [16,17] and references therein) and consists in cyanobacteria of two sequence related large subunits (PsaA and PsaB), three extrinsic subunits (PsaC–E) and a number of small intrinsic subunits (PsaF, PsaI–M and PsaX). Green plants do not contain PsaM and PsaX, but do contain three additional and larger intrinsic membrane proteins (PsaG, PsaH and PsaO) and one additional extrinsic protein (PsaN), the only extrinsic protein of PSI that is exposed to the thylakoid lumen [18–20]. The PSI core complex of *T. elongatus* binds 96 Chl *a* and 22 β -carotene molecules [1]. PSI core complexes from other organisms do not necessarily have to bind exactly the same numbers of pigments, also because the numbers of the so-called ‘red’ chlorophylls differ considerably among PSI core complexes from different organisms [21]. The PSI core complex from pea binds probably 101 chlorophyll molecules [2].

The PSI core complex occurs in trimers in cyanobacteria [22,23] and prochlorophytes [24–26], but in green plants and green algae the complex probably only occurs in a monomeric aggregation state. Dimers and larger aggregates of PSI have been observed in preparations from spinach [27], but these aggregates were induced after the solubilization of the membranes and do not represent the native complex. In cyanobacteria, the PsaL subunit has been shown to play a role in the trimerization [28]. It was suggested that the binding of the PsaH subunit to PsaL can explain the absence of trimers in green plants [2,29], because this subunit almost completely encircles the membrane-exposed part of PsaL (Fig. 2). The PsaO subunit (not resolved in the pea PSI structure) may also contribute to this effect [19]. It has been suggested that also the presence of Chl *b* can prevent the trimerization [30], but this does not hold for prochlorophytes, because these organisms form PSI trimers and contain substantial amounts of Chl *b*. In *T. elongatus*, PsaL binds 3 Chl molecules [1], while in *Arabidopsis* PsaL and PsaH together bind about 5 Chl molecules [31]. In cyanobacteria, these chlorophylls may be involved in excitation energy transfer between the monomeric units in trimers, whereas in green plants these chlorophylls are most likely involved in energy transfer from LHCII to the PSI core complex in the so-called state 2 (see Section 6.1).

The crystal structure makes clear that in cyanobacteria the PsaA, PsaB, PsaF, PsaJ, PsaK and PsaX subunits are at the periphery of the PSI trimer, and can in principle provide contacts with a peripheral membrane-intrinsic antenna complex, if present (see Section 2.4). PsaJ binds chloro-

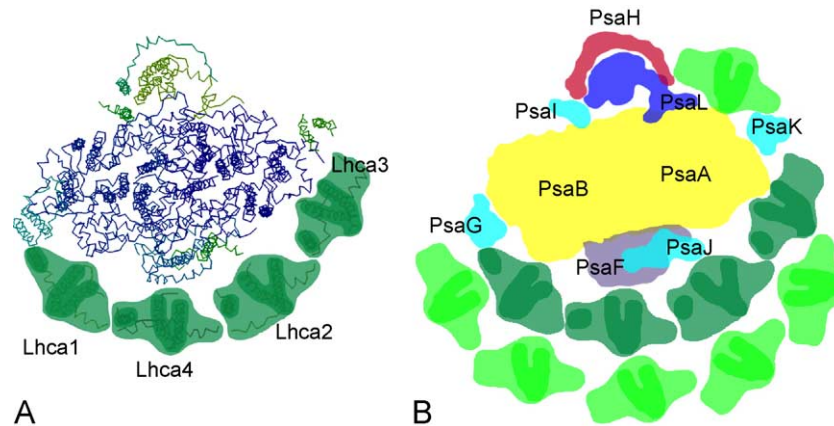


Fig. 2. Structure of photosystem I complexes. (A) Structural model of plant PSI backbone at 4.4-Å resolution (1QZV.pdb file [2]). The position of the four LHCI subunits flanking the core part is indicated with a green overlay. (B) A model of the largest determined PSI-LHCI complex of the green alga *C. reinhardtii*. Core components and LHCI subunits from the plant structure were modeled on the projected structure at 14-Å resolution determined by electron microscopy (from Ref. [48]). Fitting indicates space for 4 inner LHCI subunits, supposed to be at positions similar to those in plant PSI (dark green) plus an additional number of 4 or 5 copies in a second half-ring (5 shown here, bright green). There is also space for an additional LHCI subunit between the PsaH and PsaK subunits.

ophylls that provide part of the energy transfer route from the peripheral antenna to the PSI core complex. PsaK is at the tip of the PsaA side of the complex and has a proven connection to the peripheral antenna, because mutants without PsaK bind smaller amounts of the peripheral antenna proteins Lhca2 and Lhca3 [32]. Higher plants do not have PsaX, but do contain PsaG. The 4.4-Å structure from pea [2] suggests that PsaG is located at the tip of the PsaB side of the complex (Fig. 2). Cross-linking studies revealed no cross-links between PsaG and small PSI subunits [33], in agreement with the pea structure. An analysis of PSI particles prepared from an antisense mutant of *Arabidopsis* without PsaG suggested that PsaG is not directly involved in light harvesting [34], which was confirmed by the finding that it probably binds no or very small amounts of Chl, but 2–3 β -carotene molecules [31]. Electron transport rates were almost 50% higher without PsaG, which suggests that PsaG is involved in the regulation of the electron transport activity [34]. The 4.4-Å structure from pea [2] suggests that PsaG forms a main anchor point for the peripheral antenna, in line with the decreased stability of the binding of the peripheral antenna without PsaG [34].

2.2. Peripheral antenna

PSI of green plants and green algae binds an additional membrane-bound peripheral antenna, called LHCI. In green plants, this antenna consists of four different polypeptides from the Lhc super-gene family, called Lhca1–4, with protein masses of around 25 kDa [35]. In *Arabidopsis*, two additional genes have been identified (Lhca5 and Lhca6), but their expression is low, and it is not clear if the gene products occur in LHCI [36]. Lhca1 and Lhca4 form a heterodimer [33,37,38]. By using altered proteins produced by deletion or site-directed mutagenesis, the amino acids

required for the assembly of LHCI-730 could be identified [39]. Lhca2 and Lhca3 may also form a heterodimer [33,40,41]. Biochemical studies suggested that each Lhca protein binds 10 Chl *a* or Chl *b* molecules, as well as a few xanthophylls [41]. The recently reported 4.4-Å crystal structure [2] revealed 12 chlorophylls for Lhca1, Lhca2 and Lhca4, 11 for Lhca3, as well as 9 “linker” chlorophylls between the Lhca complexes.

The green alga *Chlamydomonas reinhardtii* contains considerably more polypeptides from the Lhc super-gene family than green plants. A recent proteomics approach revealed 18 different proteins, though some are thought to be the result of posttranslational modifications and some others occurred in very low quantities [42]. A number of 10 different subunits was suggested based on biochemical studies [43], and a recent proteomics approach revealed nine different proteins [44], consistent with structural data (see Section 2.3). Also other species of algae contain an LHCI antenna consisting of proteins from the Lhc super-gene family [45].

2.3. PSI-LHCI complexes

Electron microscopy (EM) investigations on the PSI-LHCI complex from spinach have indicated that the LHCI subunits bind in one cluster at the side occupied by the PSI-F and PSI-J subunits of the core complex [27]. The cluster is linked to the PSI core complex at positions that in cyanobacteria are excluded from the trimer interface, which suggests that the presence of the LHCI antenna does not prevent trimerization. Four masses can be distinguished in the LHCI part of the complex and it is now clear that these four masses relate to four Lhca monomers [2]. It is not known yet which mass arises from which monomer, though the suggestion of Ben-Shem et al. [2] of a sequence of Lhca1–Lhca4–Lhca2–Lhca3 (from the PsaG to PsaK side of

the complex) seems reasonable, for all because of the connection between the PsaK and Lhca2 and Lhca3 [32]. An alternative sequence of Lhca1–Lhca4–Lhca3–Lhca2 could perhaps also be possible, because then there is a symmetry of complexes with extreme red chlorophylls (Lhca3 and Lhca4—Ref. [46]) in the middle, and complexes without extreme red chlorophylls (Lhca1 and Lhca2) but with excellent connections to the PSI core at the peripheries. Spectroscopic measurements have indicated that the LHCI antenna system is well coupled to the PSI core complex and that within about 120 ps almost all excitations absorbed by LHCI chlorophylls are trapped by charge separation in PSI (see, e.g., Refs. [31,47]), which is not surprising in view of the presence of various linker chlorophylls between LHCI and the PSI core complex and between adjacent LHCI proteins [2].

PSI–LHCI complexes from *C. reinhardtii* are much larger than those of green plants [48,49]. It was found that two different types of particles exist. The larger particle has longest dimensions of 21.3 and 18.2 nm in projection [48]. The smaller particle lacks a mass at the PsaL side of the complex [48,49]. It is possible that the size difference is related to the so-called state transition (discussed in detail in Section 6.1). It was suggested that the larger and smaller particles bind 14 [48] and 11 [49] LHCI complexes, respectively, but these numbers must be reevaluated since the crystal structure [2] shows that the number of LHCI complexes in green plants is 4 instead of 8, as previously assumed. We anticipate that the crystal structure of pea PSI [2] reveals the structure of the complete complex, because the dimensions are very similar to those of isolated PSI-200 particles from spinach investigated by electron microscopy and single particles image analysis [27]. Modelling of the pea PSI structure into that of *Chlamydomonas* (Fig. 2) suggests that the number of bound LHCI complexes is 9 or 10 in *Chlamydomonas*. A biochemical analysis of PSI–LHCI supercomplexes from *Chlamydomonas* has indeed revealed nine different LHCI subunits [50]. Eight or nine of those would be at the PsaFJ side of the complex, i.e., four in similar positions as in the pea PSI crystal structure and the others in a second row flanked by PsaG and PsaK (Fig. 2). Another Lhca protein can be located at the other side of the complex between PsaL, PsaA and PsaK (Fig. 2). This area constitutes also in pea a nice binding pocket with many membrane-exposed chlorophyll molecules for a protein with the size and shape of a member of the Lhc superfamily, and it is not impossible that this pocket will be occupied by an Lhca or Lhcb protein in higher plant thylakoid membranes.

The binding of LHCI to PSI and the shape difference between the smaller and larger PSI–LHCI complexes of *C. reinhardtii* are discussed in Section 6.1.

2.4. PSI–IsiA and PSI–Pcb supercomplexes

It has been shown that the cyanobacteria *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942 produce a

supercomplex consisting of a PSI core trimer encircled by a ring of 18 IsiA or CP43' subunits [51–53] when grown under iron limitation (reviewed in Ref. [54]). The IsiA protein has no resemblance to the proteins belonging to the Lhc super-gene family, but is sequence related to the PsbC (CP43) protein of PSII [55]. If IsiA binds the same number of chlorophylls as PsbC, and if there are no PsbC chlorophylls that escaped detection in the crystal structure of *S. vulcanus* [4] or *T. elongatus* [5], then the light harvesting capacity of PSI increases by 81%. A spectroscopic analysis of PSI–IsiA complexes from *Synechococcus* PCC 7942 suggested, however, an increase of about 100%, which suggests that the number of chlorophylls in each IsiA complex is about 16 [56]. Time-resolved spectroscopy has indicated that there are efficient routes of excitation energy transfer between IsiA and PSI [57,58] and that the energy transfer from IsiA to PSI can only be modelled by assuming the presence of linker chlorophylls between IsiA and PSI [58].

A very similar complex has been found in the prochlorophyte *Prochlorococcus marinus* SS120 [25]. In this organism, the PSI core trimer is encircled by a ring of 18 Pcb proteins. The Pcb proteins are sequence related to the IsiA protein of cyanobacteria and to PsbC of PSII, despite the fact that the Pcb proteins bind not only Chl *a* (as do IsiA and PsbC) but also Chl *b* [59]. Recent research has, however, indicated that chlorophyll *b* does bind to IsiA [60] and PsbC [61] when the cyanobacterium acquired the possibility to synthesize chlorophyll *b*.

It is not a rule that 18 copies of IsiA or related proteins are needed to encircle a PSI complex. A mutant of *Synechocystis* PCC 6803 without the PsaF and PsaJ subunits (which contribute considerably to the mass at the periphery of the trimer [1,62]) binds a ring of 17 IsiA units [63]. In *Synechocystis* grown under prolonged iron stress, PSI monomers with single rings of 12 or 13 IsiA units were found as well as with double rings of 31, 33 or 35 IsiA units. Similar complexes but without a central PSI complex also occurred in significant numbers [64]. Fluorescence measurements suggested that these complexes occur as such in the thylakoid membranes. It thus appears that (partial) double rings of peripheral antenna complexes not only occur with proteins from the Lhc super-gene family (the PSI–LHCI complex in *Chlamydomonas*—see above), but also with proteins from the core complex family of antenna proteins [65].

2.5. ATPase

The ATPase synthase complex of green plant chloroplasts, also known as the F1F0-ATP synthase, belongs to the family of F-type ATP synthases. Similar types of complexes exist in prokaryotes and mitochondria. The ATP synthase enzymes have been remarkably conserved through evolution. The bacterial enzymes are essentially the same in structure and function as those from

mitochondria of animals, plants and fungi, and the chloroplasts of plants. They are all composed of three specific parts: a hydrophilic and almost spherical headpiece (F1), which is associated to a smaller membrane-bound F0 moiety via a stalk region. The stalk region consists of a central stalk plus a peripheral stator connection. Most of the mass of the F1 headpiece consists of three noncatalytic α -subunits and three catalytic β -subunits of about 55 kDa which alternate in a hexagon. Subunit γ of 35 kDa fills most of the central shaft. ATP hydrolysis by the isolated F1-ATPase drives the rotation within the central shaft. In the F1F0 complex the γ subunit is connected to an 8-kDa c subunit consisting of two membrane-bound α -helices in a hairpin. Subunit c always exists as a multimer. The number of copies of c differs between species; figures of 10, 11, 13 and 14 subunits (or hairpins) have been found [66]. The chloroplast subunit III, the equivalent of c, forms a fixed ring of 14 subunits [67].

The proton motive force over the thylakoid membrane is responsible for rotation of the c subunit multimer in intact chloroplasts. This triggers the rotation of γ and ultimately the synthesis of ATP by rotary catalysis. In order to avoid futile rotation, a second stalk, or stator, connects the F1 headpiece and F0. Subunits I, II and IV and δ of green plants are involved in the stator and an additional ϵ subunit regulates the catalytic activity by binding to γ , bringing the total number of different subunits up to nine. The mitochondrial ATPase from vertebrates has three additional small subunits plus an inhibitor protein.

In the crystal structure of bovine mitochondrial F1-ATPase determined at 2.8-Å resolution, the three catalytic β -subunits differ in conformation and in the bound nucleotide [8]. The structure supports a catalytic mechanism in intact ATP synthase in which the three catalytic subunits are in different states of the catalytic cycle at any instant. Interconversion of the states is achieved by rotation of an α -helical domain of the γ -subunit relative to the $\alpha_3\text{--}\beta_3$ subassembly. The structure of the F(1)-ATPase from spinach chloroplasts was determined to 3.2-Å resolution by molecular replacement based on the homologous structure of the bovine mitochondrial enzyme [9]. The overall structure of the α - and β -subunits was highly similar to those of the mitochondrial and thermophilic subunits. Additional small subunit (δ , ϵ) structures have been determined by NMR, but no complete ATP synthase complex has been crystallized so far. Possibly the stator is too fragile to become crystallized. Therefore, we lack information about the exact conformation of the plant subunit IV (named a in prokaryotes and mitochondria). Nevertheless, the overall positions of all subunits are rather well determined.

For a long time it was considered that the F-type ATP synthase was a monomeric membrane complex. However, using the technique of blue native gel electrophoresis, it was found that the enzyme from yeast mitochondria has a dimeric state [68]. Analysis of the subunit composition of

the dimer, in comparison with the monomer, revealed the presence of three additional small proteins. Two of these dimer-specific subunits of the ATP synthase were essential for the formation of the dimeric state. The mitochondrial ATPase dimer is not a unique large supercomplex, because other respiratory chain complexes such as cytochrome reductase (Complex III) and cytochrome oxidase (Complex IV) also form specific types of associates. A systematic search for large supercomplexes in plant mitochondria was also initiated. Blue-native polyacrylamide gel electrophoresis could separate three high-molecular mass complexes of 1100, 1500 and 3000 kDa, respectively [69]. Mass spectrometry showed that the 1100-kDa complex represented dimeric ATP synthase. This dimer was only stable under very low concentrations of detergents. However, there are no indications that the chloroplast ATP synthase forms dimers within the membrane or has specific associations with another type of large membrane complex. Image analysis of chloroplast F1 headpieces within the membrane did not reveal any specific interaction (Fig. 3; E.J. Boekema, unpublished results).

2.6. Cytochrome b_6/f complex

The cytochrome b_6/f complex is a dimeric integral membrane protein complex of about 220 kDa composed of eight to nine polypeptide subunits [70]. The four largest ones have defined functions. The 24-kDa cytochrome b_6 subunit has four transmembrane α -helices and contains two b -type hemes, together with the 17-kDa subunit IV, which has three transmembrane helices. Cytochrome b_6 and subunit IV are homologous to the N- and C-terminal halves of cytochrome b of the bc_1 complex from the respiratory chain in mitochondria. The 19-kDa Rieske iron-sulfur protein, consisting of an N-terminal single transmembrane α -helix domain and a 140-residue soluble extrinsic domain with a linker region connecting these two domains, has an overall function similar to that of the iron-sulfur protein in the bc_1 complex, deprotonating the membrane-bound quinol and transferring electrons from the quinol to the membrane-bound c -type cytochrome. The 31-kDa c -type cytochrome f subunit is functionally related to, but structurally completely different from, the cytochrome c_1 in the bc_1 complex. In addition to these four large subunits four smaller subunits, PetG, PetL, PetM and PetN, are each bound to the complex with one membrane-spanning α -helix. They have no counterparts in the cytochrome bc_1 complex.

X-ray structures at 3.0 Å of the complex from the thermophilic cyanobacterium *Mastigocladus laminosus* [10] and at 3.1 Å from the alga *C. reinhardtii* [11] have been recently obtained. The structure of the b_6/f complex bears similarities to the respiratory cytochrome bc_1 complex [71] but also exhibits some unique features, such as binding one β -carotene and one chlorophyll a , and an unexpected heme sharing a quinone site. This heme is atypical as it is

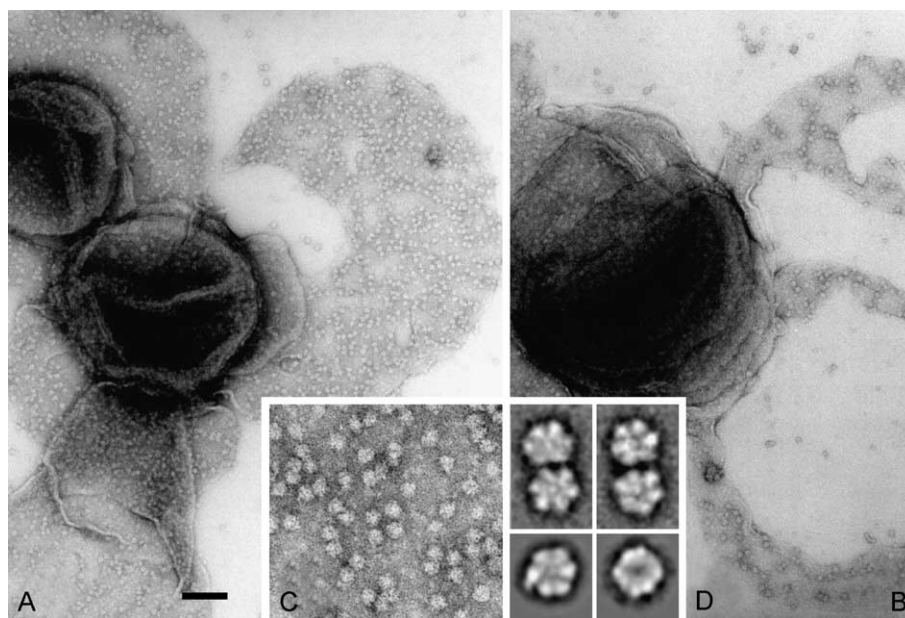


Fig. 3. Transmission electron microscopy of green plant thylakoid membranes. (A, B) Negatively stained images of detergent-free slightly disrupted spinach chloroplasts often show rather intact stacks of grana (strongly stained areas) surrounded by large areas of stroma membranes. (C) At $3\times$ higher magnification these membranes show to be associated with large numbers of protein molecules with a size around 100 Å. (D) Results of single particle image analysis of several 1000 single particles projections from intact stroma membranes. Classification indicated that about 40% consisted of ATP synthase views (lower row, left) on which the stator is visible at the left side; about 50% consisted of Rubisco (lower row, right) and 10% of F1 ATP synthase molecules that seemingly were dimeric (upper row). Further analysis of the latter group indicated that there is no specific interaction. In all classes the relative positions of both F1 headpieces were slightly different, as is also the case for the two best classes (out of 16) presented here (J.F.L. van Breemen, E.J. Boekema, unpublished results). Photosystem I, which is mostly membrane inserted, does not become well depicted, since negative stain does not penetrate the membrane. The bar in (A) and (B) equals 100 nm.

covalently bound by one thioether linkage and has no axial amino acid ligand. This heme may be the missing link in oxygenic photosynthesis [72]. The functions of the chlorophyll and β -carotene cofactors are unknown.

In the linear electron transfer scheme, the cytochrome b_6/f complex receives electrons from PSII by plastoquinol and passes them to PSI by reducing plastocyanin or cytochrome c_6 . This results in proton uptake from the stroma and generates a proton electrochemical gradient across the membrane, powering the Q-cycle and boosting ATP synthesis at the expense of reducing equivalents. Unlike the mitochondrial and bacterial homologue cytochrome bc_1 , cytochrome b_6/f can switch from linear electron transfer between both photosystems to a cyclic mode of electron transfer around PSI using an unknown pathway (see Section 5.5). Furthermore, the cytochrome b_6/f complex is thought to regulate state transitions by activating a protein kinase [73,74] (see Section 6.1).

There is no direct evidence that the cytochrome b_6/f complex can be associated to any other large complex of the thylakoid membranes [75], but the 35-kDa Ferredoxin:NADP⁺ oxidoreductase can bind with one copy. This provides the connection to the main electron transfer chain for ferredoxin-dependent cyclic electron transport [70]. It has been proposed that the cytochrome b_6/f complex forms a supercomplex with PSI, to sustain fast cyclic electron transport in the stroma (see, e.g., Ref. [80]), but there is no direct structural evidence to support this proposal. It has also

been proposed that “small plastoquinone diffusion microdomains” would exist in grana membranes [76,77], in which a few PSII complexes and a cytochrome b_6/f complex share a domain in which plastoquinone can quickly migrate between PSII and b_6/f . Such microdomains were proposed to be required because plastoquinone diffusion was estimated to be very slow in grana membranes [78,79]. About 70% of the PSII centers should be present in such domains, while the remaining PSII centers are present in (much) larger domains [77]. However, direct experimental evidence for the existence of such domains is lacking (see also Section 4.4).

3. Molecular organization of photosystem II

3.1. PSII core

The structure of the PSII core complex of the cyanobacterium *T. elongatus* is known at 3.8-Å resolution [3] and that of *Synechococcus vulcanus* at 3.7-Å resolution [4]. Recently, a refined structure at 3.5-Å resolution of PSII from *T. elongatus* was reported [5]. The complex contains four large membrane-intrinsic subunits (called PsbA–D), three membrane-extrinsic subunits (PsbO–Q in plants and PsbO, PsbU and PsbV in cyanobacteria) and a large number of small subunits, most of which span the membrane once. In all crystal structures 14 transmembrane α -helices from small

subunits are observed [3–5]. Green plants contain probably two additional small proteins that span the membrane once [81].

PsbA (D1) and PsbD (D2) bind six chlorophyll *a* and two pheophytin *a* molecules, while PsbB (CP47) and PsbC (CP43) bind 16 and 14 chlorophyll *a* molecules, respectively [5]. PsbA and PsbD constitute the photochemical reaction center in which the charge separation and primary electron transfer reactions take place [82,83], while PsbB and PsbC have a light-harvesting function, i.e., they absorb light and transfer the excitation energy to the reaction center. Even more importantly, they also accept excitation energy from the peripheral antenna and transfer this to the reaction center as well [55,84]. It is of interest to note that there are no indications yet that any of the small proteins binds chlorophyll. Some may, however, be involved in the binding of β -carotene [5]. This contrasts with the situation in PSI, in which several of the small proteins do bind chlorophyll (see Section 2.1). The extrinsic proteins do not bind chlorophyll either.

In cyanobacteria, the basic unit of PSII that was crystallized is a dimer [3–5]. The α -helices of four not yet identified small subunits as well as helices of PsbA (D1) and PsbB (CP47) are located in the dimerization domain [4] (Fig. 4B). It has been shown that the PSII monomers can be fully active [85] and that the organization of the dimers is very similar in cyanobacteria and green plants [86,87]. Also

the green alga *C. reinhardtii* [88] and the prochlorophyte *Prochloron didemni* [89] contain virtually the same dimeric structure (see also Ref. [90]). This is remarkable because the organization of the peripheral light-harvesting antenna system is very different in these types of organisms.

There has been a long-standing discussion about the aggregation state of PSII in green plants. Most evidence suggests that PSII is dimeric in the stacked, appressed parts of the membranes. In plants, the dimeric aggregation state may be stabilized by protein phosphorylation [91], the binding of phosphatidylglycerol [92], the presence of PsbW [93], PsbK and PsbL [94] or PsbO (the 33 K extrinsic protein) [95], though it is not clear whether the stabilization is a direct or indirect effect of the presence of protein or lipid. We recently discussed that the stacking of the thylakoid membranes may be important as well [96]. In unstacked thylakoid membranes no dimers could be extracted from the membranes. It was suggested that the dimers spontaneously disintegrate into monomers when they leave the grana membranes, thus facilitating the PSII repair cycle that takes place in the stromal parts of the membranes [97].

The cyanobacterial crystal structures reveal that there are three regions of the complex in which the small subunits occur. One helix from a small subunit is located quite separately from the other small proteins between PsbC (CP43) and PsbA (helix 1 in Fig. 4B), three helices are

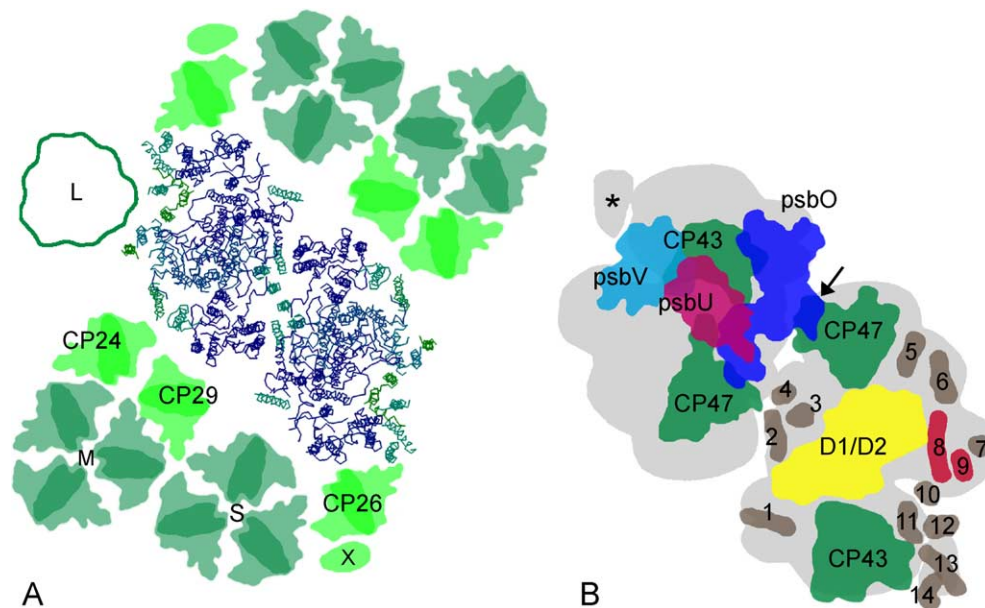


Fig. 4. (A) Top view of a $C_2S_2M_2$ supercomplex, based on electron microscopy images of PSII–LHCII supercomplexes from spinach [127]. “S” and “M” refer to strongly and moderately bound LHCII, respectively. The area marked “L” (not on scale) indicates an additional LHCII trimer only found in spinach. The central part indicates the protein backbone in the membrane-intrinsic part of the PSII core complex (calculated from the 3.7-Å structure of the PSII core complex from *S. vulcanus* (1IZL.pdb file [4])). The cross marks the place where, according to fitting and a comparison of slightly different types of supercomplexes [128], one additional small peripheral subunit could be attached. (B) Location of the membrane-intrinsic (right) and membrane-extrinsic luminal exposed (left) parts of the PSII complex. The grey area is taken from Ref. [98] and marks the PSII core area determined by electron microscopy of 2D crystals, except for the area marked with an asterisk, which is present in both the supercomplexes as well as in the X-ray structures and appears to be occupied by PsbZ [5]. The arrow marks the overlap area of the extrinsic PsbO subunit and CP47 of the adjacent core monomer. The numbers indicate the 14 transmembrane α -helices assigned to small proteins, of which numbers 8 and 9 (in red) were identified as the PsbF and PsbE protein subunits of cytochrome *b*-559, respectively.

found in the center of the dimer (helices 2–4 in Fig. 4B), while the remaining 10 (including PsbE and PsbF which together form cytochrome *b*-559) form an almost continuous row of helices along the outer rim of the complex from PsbB to PsbC (helices 5–14 in Fig. 4). This part of the complex is therefore almost free of chlorophyll (the peripheral chlorophyll molecule known as ChlZ_{D2} seems to be the only chlorophyll near the outer rim of the complex [3–5]). We note that green plants probably contain two additional small proteins that span the membrane once (PsbR and PsbW), but it is not clear where these proteins are located [81]. One of those could be located near helix 1 (Fig. 4B) [98]. It has been suggested that PsbW stabilizes the PSII dimer [93]. It is, however, unlikely that this protein occurs in the center of the dimer, because of the structural similarity of the dimeric organization in green plants and cyanobacteria, and of its chemically induced cross-link with PsbE (the large subunit of cytochrome *b*-559, helix 9 in Fig. 4B), which is located at the opposite side of the complex [99,100].

3.2. Peripheral antenna

In higher plants and eukaryotic algae, the peripheral antenna of PSII consists of a number of pigment–protein complexes belonging to the Lhc super-gene family [36]. In green plants two types of peripheral antenna proteins associated to PSII can be distinguished. The most abundant complex is the so-called “major” LHCII antenna complex. This complex occurs in a trimeric association state [101] and is not unique in composition. It consists of various combinations of three very similar proteins, encoded by the *lhcb1*, *lhcb2* and *lhcb3* genes, that usually occur in a ratio of about 8:3:1 [35]. In addition, there are three “minor” antenna complexes, which are called Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24) and usually occur in monomeric aggregation states. All these complexes bind various molecules of chlorophyll *a* and chlorophyll *b* and of the xanthophylls lutein, violaxanthin and neoxanthin [84,102]. The structure of the major trimeric LHCII complex is known at 2.72 Å [7]. It is generally believed that the minor complexes adopt rather similar three-dimensional organizations [35,103,104].

A special case is given for PsbS. This very hydrophobic protein also belongs to the Lhc super-gene family and has four transmembrane α -helices, one more than most other members from this family. This protein has by some authors been considered as a PSII core protein (see, e.g., Ref. [105]), but it has never been found in PSII crystals. It has also been considered as a light-harvesting protein, but also this is controversial, because it is not certain whether or not this protein binds chlorophyll [84,106]. Fact is that unlike most other members of the Lhc super-gene family, this protein is stable in the absence of chlorophylls and carotenoids [107] and that it is directly involved in one of the most important regulation mechanisms of photosynthesis, the mechanism

by which excess excitation energy is harmlessly dissipated into heat [108] (discussed in more detail in Section 6.2). It has been suggested that a dimer-to-monomer conversion of PsbS accompanies this nonphotochemical quenching of excess absorbed excitation energy [109].

3.3. PSII–LHCII supercomplexes

3.3.1. General features

A variable number of the peripheral antenna proteins can associate with dimeric PSII core complexes to form the so-called PSII–LHCII supercomplexes. Supercomplexes were first recognized in electron microscopic images by their peculiar rectangular shape after a mild detergent solubilization of grana membranes [87]. These rectangular supercomplexes, in the following denoted as ‘standard’ or C₂S₂ supercomplexes (see below), contain almost all PSII core proteins [98], but from the peripheral antenna only the Lhcb1, Lhcb2, Lhcb4 and Lhcb5 gene products could be detected [110]. The PsbS protein is absent as well [111], though the absence of PsbS in PSII–LHCII supercomplexes is not generally accepted [112]. A three-dimensional structure of this supercomplex was constructed (at 24-Å resolution) by single-particle analysis of images obtained by cryoelectron microscopy [113]. It was shown that the three-dimensional structures of PSII–LHCII complexes from the green alga *C. reinhardtii* [88] and the liverwort *Marchantia polymorpha* [114] are very similar to that of higher plants. Combining this work with the X-ray crystallography work on cyanobacterial PSII [3] revealed quite some detail of the structure of the core part of the PSII–LHCII supercomplex [90].

An important question is whether or not the (rectangular) PSII–LHCII supercomplex represents the native organization of PSII in the grana membranes. Some authors [115] suggested that the dimeric supercomplex represents an artefact induced by the solubilization of the membranes. We do not share this opinion and note that rectangular supercomplexes were not only observed after detergent solubilization of PSII grana membranes, but also (and with very high yield) after detergent solubilization of complete thylakoid membranes [116]. In addition, EM micrographs of partially unfolding grana membranes clearly revealed the presence of rectangular supercomplexes in the membranes [117], which indicates that the supercomplexes occur as such in the membranes. More evidence for the occurrence of PSII–LHCII supercomplexes in grana membranes has been provided by analyses of regular arrays of particles in grana membranes and of mutants of *Arabidopsis thaliana* that express antisense constructs (see Section 4).

An important consequence of the organization of the PSII–LHCII supercomplexes is that the Lhcb4–LHCII–Lhcb5 antenna structure needs a dimeric PSII core to become attached to PSII. Lhcb4 (CP29) binds to one PSII core monomer, Lhcb5 (CP26) to the other, while the

trimeric LHCII unit has clear contacts with both Lhcb4 and Lhcb5 (Fig. 4A). Indeed, monomeric cores with attached LHCII have never been observed in mixed populations of disrupted grana. This also explains why the peripheral antenna proteins easily detach from PSII during the transition from dimer to monomer. Our analyses did not reveal other types of association of PSII and LHCII than the supercomplex. Other associations can, however, not be completely ruled out if one looks at positions of core complexes within the membrane [118]. These authors claimed a rearrangement of the LHCII antenna proteins around the core dimer, based on the finding of a new type of crystal packing. However, after testing the packing of this lattice with the standard C_2S_2 supercomplexes, we conclude that such particles nicely can fit within the published lattice and that the proposed LHCII rearrangement is an over-interpretation of low-resolution data.

3.3.2. Extrinsic proteins

After removal of extrinsic proteins by Tris-washing PSII–LHCII supercomplexes could be observed with either an elongated or a shortened appearance [95]. These types of supercomplexes could not be detected in the databases of supercomplexes obtained from oxygen-evolving or salt-washed PSII membranes, even when an elongated or shrunk supercomplex was used as reference in the data analysis. It was suggested that the removal of PsbO (the extrinsic 33-kDa protein) causes a diminishing of the interaction between the two PSII core units. It was also shown that removal of PsbO and/or PsbP (the 23-kDa protein) can induce a displacement of LHCII and/or Lhcb4 towards the PSII core complex [95]. This suggests that the extrinsic proteins not only have roles in the cofactor requirement for photosynthetic oxygen evolution [119], but also may function to keep the peripheral antenna at a proper distance to maintain sequestered domains of inorganic cofactors required for oxygen evolution. The PSII structure from *S. vulcanus* [4] provides some evidence for this. The PsbO protein has a number of close contacts with the CP47 subunit of the other PSII monomer and also extends over the outer rim of the complex at a position where in green plants trimeric LHCII is bound (Fig. 4B).

We note that the position of the PsbO protein in the structures of the cyanobacterial PSII core complex [3–5,120] differs from that anticipated earlier for the PSII core complex from green plants [113,121,122] (see also Ref. [123]). In this earlier view, the PsbO protein was thought to be located at the most stain-excluding region of the PSII core complex near the “C” of CP47 in the left part of Fig. 4B, both in green plants and in cyanobacteria. This view was based in part on the amount of staining in top views in electron microscopy images, which appeared to be very similar for PSII in green plants and cyanobacteria. The most stain-excluding area of the cyanobacterial PSII core complex is at a position with, according to the recent structures, almost no extrinsic mass, so it seems that the extrinsic

proteins have only minor effects on the staining of the PSII core complexes.

A matter of current debate is the number of PsbO proteins per PSII monomer. The crystal structures of cyanobacterial PSII core complexes reveal only one copy [3–5], but binding studies of PsbO to plant PSII suggest binding of two copies with markedly different binding affinities [124]. A recent study on the binding domains of PsbO from spinach suggested two different binding domains, one of which is absent in the amino acid sequences of cyanobacterial PsbO [125]. It is possible that a second PsbO protein is present in green plant PSII, provided that it has minor effects on the staining of PSII as seen in top views in electron microscopy images.

3.3.3. Binding of trimeric antenna proteins

The rectangular supercomplex contains two trimeric LHCII complexes per PSII core dimer. However, the total number of trimeric LHCII per PSII dimer is generally about eight, which immediately raises the question of the location of the remaining population of LHCII trimers. Based on the shortest (<5 min) and mildest (using the nonionic detergent *n*-dodecyl- α ,D-maltoside, α -DM) possible way of solubilizing PSII grana membranes from spinach, we found by electron microscopy and single particle analysis of partially solubilized complexes two additional binding sites for trimeric LHCII at the PSII core dimers [126–128]. According to the frequency of occurrence the three binding sites were designated “S”, “M” and “L” (from strongly, moderately and loosely bound LHCII, respectively) (Fig. 4A). Analysis of the averaged images of $C_2S_2M_{1-2}$ supercomplexes showed that the M trimer is rotationally shifted by about 20° compared to the S trimer [128]. In *Arabidopsis thaliana*, only S and M trimers were found, but in the same position [129]. However, the M trimers were more abundant in *Arabidopsis* than in spinach (see also below). Also in the liverwort *M. polymorpha* the M trimers are more abundant than in spinach [114].

The S-LHCII trimer consists predominantly of the Lhcb1 and Lhcb2 gene products, because only these were detected in C_2S_2 supercomplexes [110]. In contrast, the M-LHCII trimer consists most likely of the Lhcb1 and Lhcb3 gene products, because the Lhcb3 gene product is present in the larger supercomplexes [127] and because it occurs in a supercomplex consisting of the Lhcb1, Lhcb3, Lhcb4 and Lhcb6 gene products in a 2:1:1:1 ratio [130,131]. The M-LHCII trimer and the Lhcb4 and Lhcb6 gene products are very close together in the PSII–LHCII supercomplex (Fig. 4A). This suggests that one of the constituents of M-LHCII is encoded by Lhcb3. The positions that Lhcb1 and Lhcb3 may occupy have not yet been determined.

Analysis of supercomplexes isolated from *Arabidopsis* plants expressing an antisense construct to Lhcb2 revealed that the LHCII binding sites are not unique for the various types of trimers [132]. In these plants, not only the synthesis of Lhcb2 was almost completely abolished, but also that of

the strongly related Lhcb1 protein [133]. It appeared that in these plants, the expression of the antisense Lhcb2 construct resulted in strongly increased levels of Lhcb5 (CP26) and (to a minor extent) Lhcb3, and that supercomplexes were formed with trimers consisting of Lhcb5 and Lhcb3 at the S- and M- binding positions [132]. This replacement is unique, because expression of antisense constructs to the minor peripheral antenna proteins and to the peripheral antenna proteins of PSI did not lead to increased synthesis of other proteins (discussed in detail below), and stresses the importance of the particular organization of PSII and LHCII in supercomplexes.

3.3.4. Binding of monomeric antenna proteins

Our work also gave new information on the location and binding of the minor complexes Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24). Based on cross-linking experiments [134], it was assumed that Lhcb5 is located near PsbC (CP43) on the tip of the PSII–LHCII supercomplex, whereas Lhcb4 is located near PsbB (CP47) on the other side of the complex (Fig. 4A). A recent structural analysis of supercomplexes prepared from antisense mutants of *Arabidopsis* without Lhcb5 or Lhcb4 confirmed this view [135]. Lhcb6 is absent in the ‘standard’ C₂S₂ supercomplex, but present in the larger complexes [127,134], which strongly suggests that Lhcb6 represents the additional mass with the size of a monomeric light-harvesting complex close to the M trimer and Lhcb4 (Fig. 4A). A physical interaction between Lhcb6 and Lhcb4 became also obvious from *Arabidopsis* plants with antisense constructs to Lhcb4 or Lhcb5 [136].

The different minor complexes seem to have unique and different roles in the supramolecular organization of PSII and LHCII. Supercomplexes with empty Lhcb5 binding site but with otherwise normal appearance could be isolated from *Arabidopsis* plants expressing antisense constructs to Lhcb5 [135]. In addition, classification of projections of supercomplexes from spinach [95,128] or *Arabidopsis* [129] suggested the existence of many supercomplexes with empty Lhcb5 binding sites. This indicates that Lhcb5 is not needed for the formation of PSII–LHCII supercomplexes and that antisense removal of this protein does not lead to replacement by one of the other members of the Lhc super gene family. No intact supercomplexes could be isolated from *Arabidopsis* plants expressing antisense constructs to Lhcb4 [135], and PSII–LHCII supercomplexes with empty Lhcb4 binding sites could not be found [128]. This suggests that also Lhcb4 occupies a unique position in the PSII macrostructure and that, in contrast to Lhcb5, its presence is essential for the formation of PSII–LHCII supercomplexes. Both the Lhcb4 and Lhcb5 antisense mutants showed a rather normal photosynthetic performance, although the mutants showed slightly different fluorescence characteristics and an increased number of PSII centers [133]. This suggests that the organization of PSII and LHCII into supercomplexes is not absolutely required for photosynthetic performance, at least under

normal physiological conditions and light levels. In the field, however, most mutants showed decreased ecological flexibility, an important parameter for plant fitness under natural conditions [137,138].

3.3.5. Role of small PSII core subunits

Of special interest for the supramolecular organization of the PSII core complex and its surrounding peripheral antenna are those small subunits of the PSII core that could play a role in the binding of the peripheral antenna. The constructed figure of the PSII–LHCII supercomplex suggests that of the 14–16 small proteins only a few seem to be involved in contacts with the peripheral antenna. The first is the one (or two in green plants) protein located between PsbA and PsbC (helix 1 in Fig. 4B). This protein may contribute to the binding of S-LHCII and may therefore be important for the supramolecular organization of PSII and LHCII. In the structure of *S. vulcanus* this protein remained unassigned [4], whereas in the structure of *T. elongatus* it was assigned to PsbI [5]. It is possible (but by no way proven) that the PsbR protein (not present in cyanobacteria—see Section 3.1) is also located at this position. Two other proteins (helices 5 and 6 in Fig. 4B, attributed to PsbH and PsbX in [5]) seem to have contacts with CP24.

Another protein that may be involved in the association of the peripheral antenna is the protein located at the outer tip of PsbC in the supercomplex (helices 13 and 14 in Fig. 4B). It is possible that this protein is PsbZ (also known as Ycf9 or Orf62), the only small protein in PSII that has two transmembrane α -helices, because mutants without PsbZ appeared to have strongly reduced Lhcb5 levels [139,140]. The location as indicated in the figure is consistent with the reduced level of Lhcb5. However, it appeared that without PsbZ no PSII–LHCII supercomplexes could be isolated, which is not consistent with results from an *Arabidopsis* mutant with an antisense construct to Lhcb5 [135]. This and other studies revealed that Lhcb5 is not required for the stabilization of PSII–LHCII supercomplexes. Another possibility is that helix 14 arises from PsbJ, because also without this small protein no stable PSII–LHCII supercomplexes could be detected, whereas it also seems to interact with the extrinsic proteins PsbP and PsbQ [141], which are expected near this position (Fig. 4B). Ferreira et al. [5] assigned helix 10 to PsbJ, but this location is far from the interaction domain with the peripheral antenna, and does not easily explain the absence of supercomplexes if PsbJ is absent.

If PsbZ is not responsible for helices 14 and 13, it can also be located at the first interaction position (see above), near helices V and VI of PsbC and helices A and B of PsbA (helix 1 in Fig. 4B). Also two chlorophylls of PsbC and one of PsbA (ChlZ_{D1}) are positioned near this helix, so this protein could be important for energy transfer from the peripheral antenna to the PSII core. A location of PsbZ at this position is consistent with the absence of super-

complexes, and can be consistent with the absence of Lhcb5 if the absence of S-LHCII prevents the binding of Lhcb5. In green plants two helices are modeled at this position [90], but in cyanobacteria only one [3–5]. The amino acid sequence of PsbZ from *Synechocystis* suggests two helices [81], which is inconsistent with a location of PsbZ at helix position 1 in the structural models of the cyanobacterial PSII core complex. We conclude that more studies are required to understand the role of PsbZ in the PSII–LHCII interaction.

3.3.6. Energy transfer

The constructed figure (Fig. 4A) gives some indication on the possible energy transfer routes in the supercomplexes. CP43 (PsbC) can receive excitation energy from both CP26 (Lhcb5) and S-LHCII. At the present resolution it is impossible to estimate the distance between the most nearby chlorophylls, but nevertheless it seems reasonable to assume efficient energy transfer along these routes. CP47 (PsbB) has excellent contacts with CP29 (Lhcb4). The distance between CP24 (Lhcb6) and CP47 seems slightly larger, so most energy transfer from CP24 may go via CP29. Interestingly, there seems to be a rather large distance between the chlorophylls of L-LHCII and of the PSII core complex, because L-LHCII is located behind the rim of chlorophyll-free small subunits of the PSII core complex (Fig. 4A). This suggests that energy transfer from L-LHCII proceeds via CP26 or CP24. The only chlorophyll that comes somewhat close is the peripheral chlorophyll of PsbD (D2), so a function of this chlorophyll could be to mediate energy transfer from L-LHCII to heart of the PSII core complex. The peripheral chlorophyll of PsbA (D1) also seems quite remote from the peripheral antenna system, the S-LHCII trimer is the most nearby, but this complex has much better contacts with CP43. For more details on the energy transfer characteristics, we refer to a recent review [84].

3.4. LHCII aggregates

Most PSII–LHCII supercomplexes contain two, three or four LHCII trimers. Very few complexes with five LHCII trimers could be detected, but complexes with more trimers were never observed, despite a very large data set of EM projections of supercomplexes [127]. However, there are usually about eight LHCII trimers per PSII core dimer [130], so there is considerably more LHCII than present in the PSII–LHCII supercomplexes. This implies the presence of a pool of non-bound or very loosely bound LHCII, in line with many earlier suggestions (see, e.g., Ref. [142] and Section 4.3). Classification of single-particle projections of partially solubilized PSII grana membranes revealed small amounts (1–5%) of a very characteristically shaped particle (Fig. 5) that was identified as a heptamer of LHCII trimers [143]. The same or similar LHCII aggregates were purified by Peter and Thornber [130] and Ruban et al. [144].

3.5. PSII–Pcb supercomplexes

Prochlorophytes contain a Chl *a/b* binding protein called Pcb that does not belong to the Lhc super-gene family, but instead is related to PsbC (CP43) [59]. Recently, PSII–Pcb supercomplexes from *P. didemni* and *Prochlorococcus* MIT 9313 were described. These complexes consist of a PSII core dimer, organized in a similar way as in cyanobacteria and green plants, flanked by five Pcb subunits at each side of the dimer in *Prochloron* [89] or by four PcbA subunits at each side of the dimer in *Prochlorococcus* [26]. The Pcb subunits occur at similar positions as the Lhcb4–LHCII–Lhcb5 subunits of green plants and thus also need a PSII core dimer as a scaffold to bind rows of four or five Pcb subunits.

4. Organization of supercomplexes in stacked grana membranes

4.1. Crystalline arrays

Crystalline 2D arrays of PSII have been observed decades ago after freeze-etching and freeze-breaking of green plant photosynthetic membranes (see Refs. [105,123,145] for recent reviews). It was noted that PSII in such arrays is dimeric (see, e.g., Refs. [146–148]), but details of the molecular composition of PSII in these rows could not be provided because of the limited resolution of the freeze-etching technique. In addition, there appeared to be quite some variation in the size of the repeating unit in these rows, which suggests that several types of complexes can form repeating units, depending on factors like plant species, growth conditions, preparation method, etc.

Recently, it became possible to see details of the area between the rows, using electron microscopy and image analysis of negatively stained grana membranes isolated from spinach thylakoids after a short treatment with α -DM [149]. Two types of rows were found, called “small-spaced” and “large-spaced” rows, indicating a spacing of rows of 23 and 26 nm, respectively. The small-spaced semi-crystalline macrodomains occurred in about 1% of the membranes, and were shown to consist of a C_2S_2 repeating unit (see also Table 1), while the large-spaced semi-crystalline macrodomains occurred in about 50% of the membranes and were suggested to consist of an asymmetric C_2S_2M repeating unit [149]. The unit cell of the latter and most abundant regular arrays (27.3×18.3 nm, angle 74.5° , area 481 nm²) resembles the unit cell of a frequently occurring regular array in freeze-fractured thylakoid membranes from spinach (26.5×18.7 nm, angle 69° , area 462 nm²) [147], which suggests that the regular arrays have not been induced by the detergent treatment or by the negative stain used to image the membranes by EM (discussed in more detail in Section 4.4).

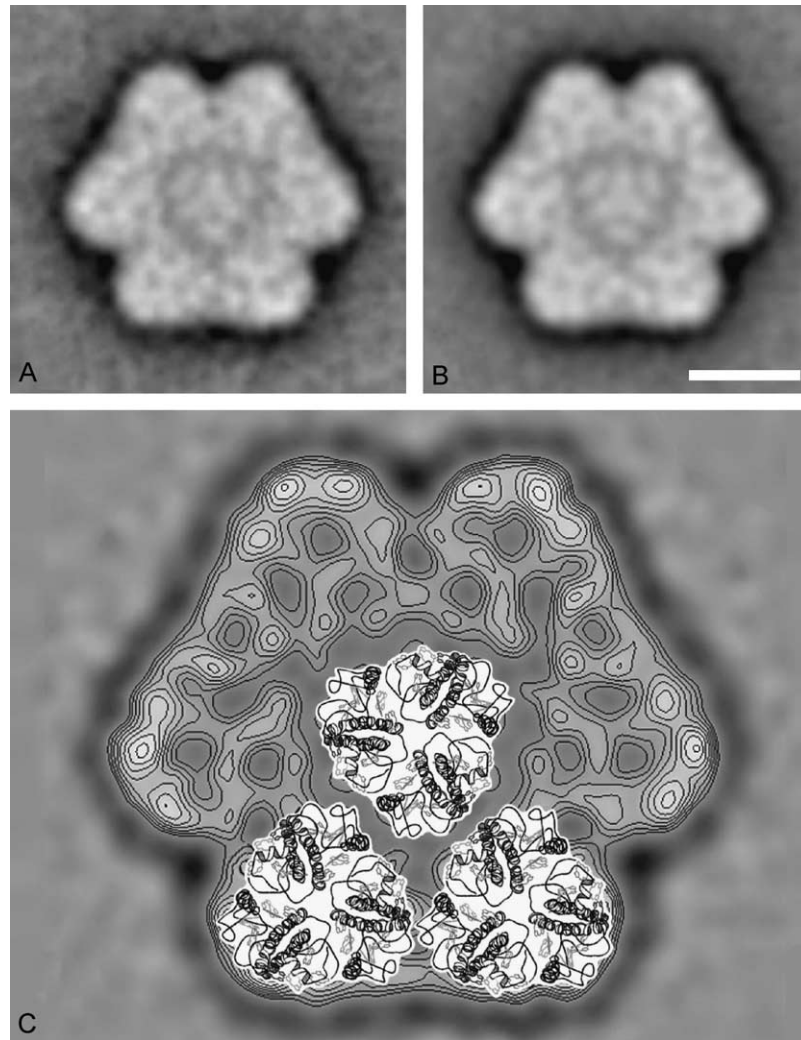


Fig. 5. The LHCII icosienamer. (A) Average image of 133 aligned complexes indicates the presence of seven LHCII trimers [143]. (B) Threefold symmetrized projection of image A. (C) Fitting of the LHCII structure [6] in the lower three densities. The bar is 10 nm.

Grana membranes from *Arabidopsis thaliana* prepared in the same way as described above for spinach consistently showed semi-crystalline macrodomains with larger unit cells than in spinach [129]. In wild-type *A. thaliana*, the unit cell was 25.6×21.4 nm (angle 77° , area 534 nm²). Image analysis revealed that these semi-crystalline macrodomains are built up from $C_2S_2M_2$ supercomplexes. The additional M-LHCII in the unit cell of *A. thaliana* compared to spinach

is consistent with the more frequent occurrence of M-LHCII in supercomplexes from *A. thaliana* than from spinach, in line with the idea that the repeating unit in these semi-crystalline arrays is a PSII–LHCII supercomplex. Membranes from the npq4 mutant of *A. thaliana*, which lacks the PsbS protein [108], revealed an identical unit cell as in the wild-type [150], which strongly suggests that the relatively large PsbS protein is not present in the regular arrays in grana membranes of wild-type plants. A slightly different unit cell was found in membranes from plants with an antisense construct to Lhcb2 [132], which can be explained by the fact that the trimers in these membranes consist of Lhcb5 and Lhcb3 instead of Lhcb1 and Lhcb2. A smaller unit cell was also found in membranes from plants with an antisense construct to Lhcb5 [135], in which case the image analysis indicated a supramolecular organization without Lhcb5 but otherwise similar to that of the wild-type. No regular arrays were found in membranes from plants with an antisense construct to Lhcb4, but PSII–LHCII supercomplexes were not found either [135]. Table 1 summarizes the

Table 1
Unit cell and composition of regular arrays of PSII and LHCII in grana preparations

	Regular arrays?	Unit	Area (nm ²)	Protein replaced?	Reference
Spinach	yes	C_2S_2	389	–	[149]
Spinach	yes	C_2S_2M	481	–	[149]
<i>Arabidopsis</i>	yes	$C_2S_2M_2$	534	–	[129]
CP29	no	–	–	no	[135]
CP26	yes	$C_2S_2M_2$	500	no	[135]
Lhcb1+2	yes	$C_2S_2M_2$	524	yes	[132]
PsbS	yes	$C_2S_2M_2$	531	no	[150]

properties of the regular arrays observed in the investigated types of plants.

These results suggest that the basic unit of PSII and LHCII in grana membranes differs in spinach and *Arabidopsis*. In spinach, the basic unit is C_2S_2M , in which two copies of S-LHCII, Lhcb4 and Lhcb5 and one copy of M-LHCII and Lhcb6 are attached to a PSII core dimer, whereas in *A. thaliana* the basic unit is $C_2S_2M_2$, in which two copies of S-LHCII, M-LHCII, Lhcb4, Lhcb5 and Lhcb6 are attached (see also Fig. 4A). In view of the occurrence of supercomplexes, it is likely that the basic unit is also $C_2S_2M_2$ in *M. polymorpha* [114]. We note that the protein mass of the $C_2S_2M_2$ unit is almost 1.1 MDa and that this complex binds approximately 190 Chl *a*, 80 Chl *b* and 75 carotenoid molecules [84].

4.2. Megacomplexes

Image analysis of PSII–LHCII supercomplexes has indicated that the supercomplexes can laterally associate to each other in rather specific ways (Fig. 6). In spinach three types of megacomplexes (dimers of supercomplexes) have been observed thus far (Fig. 6B, C and E [127,128]), while in *Arabidopsis* a fourth type was found (Fig. 6F [150]). A fifth type was recently observed in *Chlamydomonas* (Fig. 6D; A.E. Yakushevskaya, unpublished results). We note that

supercomplexes also have a tendency to form ‘sandwiches’, in particular when they were purified by sucrose density gradient centrifugation and when the carbon-coated grids used for EM were not glow-discharged to enhance hydrophilic interaction [121], but we do not consider these as megacomplexes.

The question arises whether or not the megacomplexes have any relationship with the semicrystalline arrays in the grana membranes. If there is such a relationship, then it may be possible to determine the regions of the supercomplex that are important for the formation of these arrays. The type II megacomplex of spinach (Fig. 6E) consists of two C_2S_2M complexes associated along their long sides in such a way that there is no vertical displacement of the complexes [127]. Regular arrays of a type II megacomplex were not found in our membranes, but the unit cell (26×18 nm, angle 90° , area 468 nm²) found in grana membranes from maize [151] may very well consist of a type II basic unit. The most common type I megacomplex (Fig. 6B) has a vertical displacement of 7.5 nm between the two units, which is identical to the vertical displacement in the “large-spaced” crystals [149]. The horizontal displacement between the units was about 1.5 nm larger in the megacomplexes than in the regular arrays, which can be explained by the alternating absence and presence of M-LHCII between the units in the arrays and the complete presence of M-LHCII between the

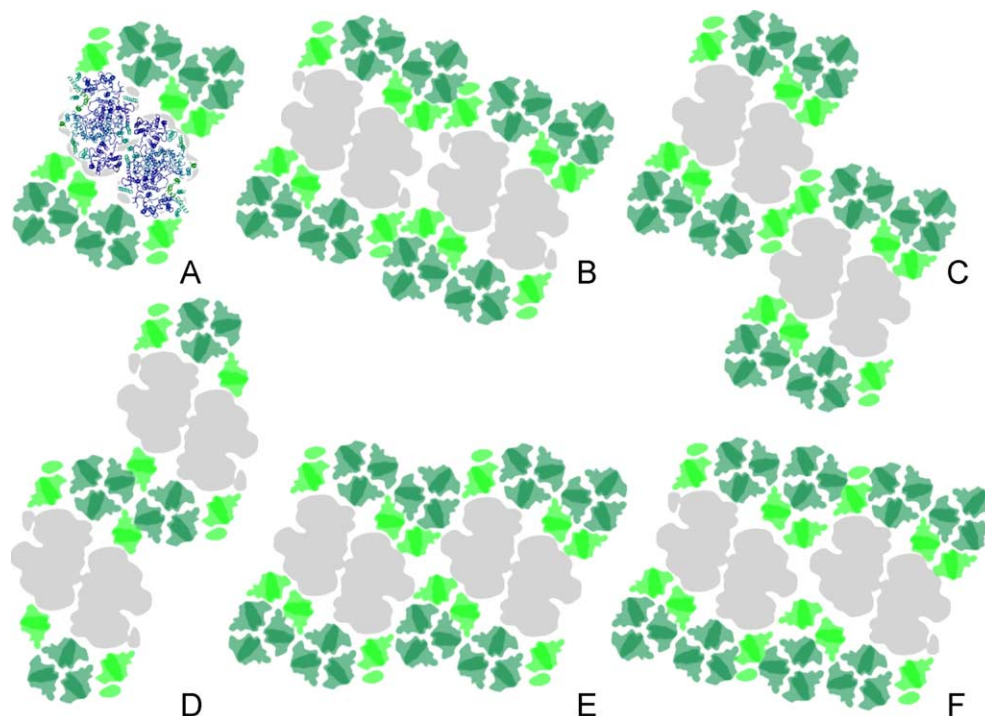


Fig. 6. Different types of PSII–LHCII megacomplexes found in green plants and green algae. (A) Top view of a $C_2S_2M_2$ supercomplex, as presented in Fig. 4A. (B) Type I megacomplex from spinach [127]. (C) Type III megacomplex from spinach and *Arabidopsis* [128,129]. (D) Type V megacomplex from *C. reinhardtii* (A. Yakushevskaya, unpublished). (E) Type II megacomplex from spinach [127]. (F) Type IV megacomplex from *Arabidopsis* [150]. The megacomplexes have been modeled from electron microscopy 2D projection maps obtained by single particle averaging. Fitting indicated that some structures only matched after omission of the PsbZ area (see Fig. 4), indicating that differences in the interfaces between two supercomplexes might be more complex than just the result of lateral displacements. The megacomplex of (D) is the only one which excludes the presence of M trimers; in the megacomplex of (C) the M trimers are partly present.

units in the megacomplexes. The minor type III megacomplex from spinach (Fig. 6C [127]) has a vertical displacement of 20 nm and appears to be the repeating unit of the regular arrays in *Arabidopsis* [129]. However, the megacomplex observed in *Arabidopsis* (Fig. 6F [150]) does not form the basis of a known regular array.

The various structures suggest that the presence of L-LHCII would prevent the formation of four of the five types of megacomplexes observed thus far, while M-LHCII in plant megacomplexes is either required for the formation of these megacomplexes (Figs. 6B, E, and F) or does not impose a steric hindrance (Fig. 6C). M-LHCII would impose steric hindrance for the formation of the megacomplex observed in *Chlamydomonas* (Fig. 6D), but this type of LHCII was not observed at all in *Chlamydomonas* supercomplexes (A.E. Yakushevskaya, unpublished observations), which may be related to the absence of an Lhcb6 homologue in *Chlamydomonas* [44,152]. It is possible that L-LHCII is bound to supercomplexes located at the end of the regular arrays. The data suggest that Lhcb5 is essential for the formation of the type I and type III megacomplexes (Figs. 6B and C), and thus for the most common regular arrays in spinach and *Arabidopsis*, respectively. On the other hand, Lhcb4 is not involved in the formation of megacomplexes, because it is located more in the interior of the supercomplex, but is essential for the formation of the supercomplexes themselves.

4.3. Organization of PSII and LHCII in opposing membranes

An advantage of the EM technique applied to negatively stained grana membranes is that both layers of the grana can be investigated simultaneously. This is not possible with other structural techniques, such as atomic force microscopy (AFM) and freeze fracture and freeze-etching EM techniques. So with EM and additional image analysis, it is possible to study the relation between the complexes in the opposing membranes in terms of the positioning of the PSII core dimer and the LHCII units.

We recently presented a detailed study of α -DM prepared grana membranes from spinach [117,149]. There appeared to be quite some heterogeneity in the number and organization of the PSII complexes in these membranes. Although all grana membranes consisted of two membrane layers, some appeared to have a much lower density of PSII complexes than others. This means that there is a large variability in the number of LHCII antenna complexes per PSII within a grana or between grana. A small part of the membranes contained regular arrays in both layers [149], and in these membranes the ratio of LHCII to PSII will be not much higher than 1.5, the number for the C_2S_2M repeating unit. Some other grana contained surprisingly little PSII [117], and the ratio of LHCII to PSII is in these membranes probably much higher than 4 (the average ratio of LHCII to PSII in grana

membranes). The origin and significance of this heterogeneity is not yet understood.

It appeared that in those grana that have rows in both membranes, there are preferential angles of rows in opposing membranes with respect to each other, both in spinach (Fig. 7) and in *Arabidopsis* (Fig. 8). At these angles the overlap of LHCII trimers is optimized, at least for the central part of the domains. For spinach the preferential angles were $3^\circ (\pm 4^\circ)$ and $46^\circ (\pm 10^\circ)$ [149]; for *Arabidopsis* they were $32^\circ (\pm 2^\circ)$ and $58^\circ (\pm 3^\circ)$ [129]. The differences are most likely related to the different basic units. These results indicate that the organization of the complexes in one membrane affects the organization of complexes in the opposing membrane.

Because the PSII–LHCII supercomplexes have a very pronounced handedness, it is possible to judge if a certain PSII complex in a granal membrane is located in the upper membrane or in the lower membrane. The handedness was analyzed in a relatively well-ordered membrane from spinach [149], and it appeared that in one part of the grana most PSII complexes were located in the upper layer, in another part in the lower layer, while only in the middle a region occurred with a row in both layers. Based on this analysis, it was proposed that most (but certainly not all) PSII–LHCII supercomplexes face an LHCII-only region in the opposing membrane (Fig. 7). A variation of this model was proposed by Ford et al. [115], who suggested that one layer would consist entirely of PSII and the other entirely of LHCII. We consider this possibility as less likely, not only because this model does not accept the PSII–LHCII supercomplex as basic unit for PSII in the membranes, but also because this model needs a much larger amount of

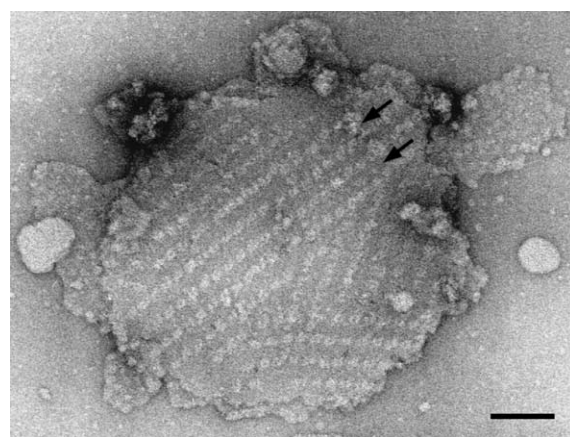


Fig. 7. Crystallinity of C_2S_2M complexes in paired inside-out grana membranes from spinach. In one of the membrane layers, the one which is stronger contrasted with negative stain; rows of crystalline core parts are visible. In the other membrane layer, the crystallinity in the largest domain is partly in the same direction (see arrows), but only a few complexes are present, indicating that LHCII dominates in these areas. On the left and right sides of the grana membrane single membrane layers are attached with a different granularity, likely caused by aggregated PSI complexes. The bar equals 100 nm.

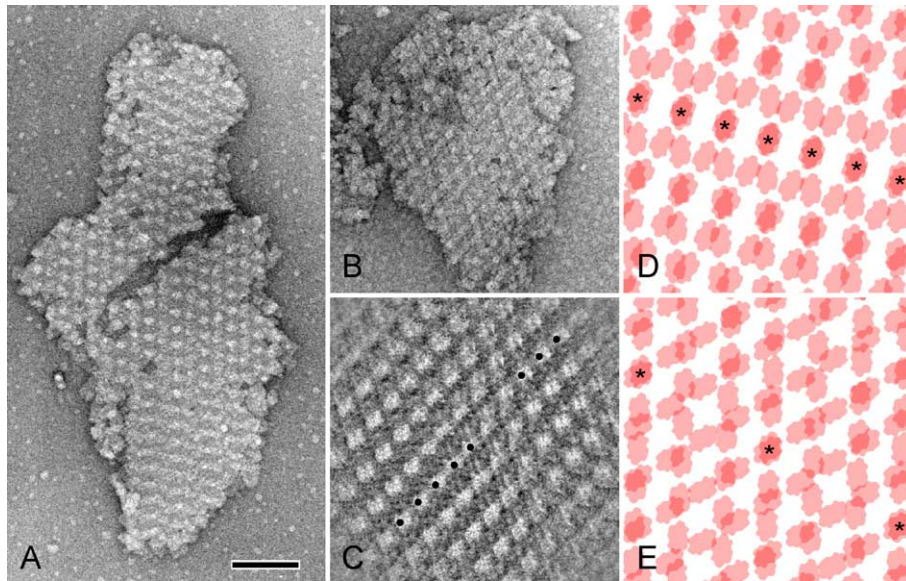


Fig. 8. Preferential stacking as determined in paired inside-out grana membranes from *A. thaliana*. If two crystalline arrays face each other they are arranged in such a way that there is optimal overlap of LHCII trimers (and hence also from core complexes) in at least the center of the sandwiched crystals. (A) Electron micrograph of a negatively stained grana fragment of a 33°-type crystal in which the two layers differ about 33° in their rotational orientation [129]. (B) Image of a grana fragment of a 58°-type in which the layers differ about 58°. (C) Fourier filtering of the inner part of the 58°-type crystal from B. The position of the black dots on the core complex densities indicates dislocation in one of the layers; possibly to get a better match between LHCII complexes from adjacent layers. (D, E) Simulation of the overlap pattern in the 33°-type and 58°-type crystals, respectively, based on 150 averaged crystals each. Asterisks indicate positions where core complexes (in red) of adjacent layers match optimally. The bar in A is 100 nm.

reorganization of PSII and LHCII if a transition occurs from a random organization to an organization in rows.

4.4. Do rows of PSII–LHCII supercomplexes represent native structures?

It is important to know if PSII in semi-crystalline arrays represents a fully functional photosystem or an inactive complex. Freeze-fracture studies have indicated that rows are largely absent in freshly prepared thylakoid membranes but can be generated by prolonged storage at 4° C [153] or by incubation in certain buffers [147,154]. It appeared that thylakoids with rows of PSII are thermally more stable than those without rows of PSII [154]. So it is possible that the organization of PSII and LHCII in rows represents an *in vivo* situation under at least some physiological conditions. Cold acclimation could be one of those conditions [155].

However, even if an organization of PSII and LHCII in rows would occur *in vivo* under some physiological conditions, it is by no way certain that this organization represents a fully functional photosynthetic unit. In such a unit, not only efficient excitation energy transfer and primary electron transport would have to occur, but also the transfer of reduced plastoquinone to the cytochrome b_6/f complex needs to be fast and effective. It has been proposed [76,77] that PSII centers with a functional plastoquinone pool can only be found in “small plastoquinone diffusion microdomains” (see Section 2.6). Such microdomains cannot exist in the regular arrays of PSII and LHCII if they have the same composition in our b_6/f -free α -DM derived

grana membranes [117] and in thylakoids after prolonged storage at 4° C [153] or by incubation in certain buffers [147,154]. Also the image analysis of the rows did not give any indication for the presence of the cytochrome complex. So, if the concept of the small plastoquinone diffusion microdomains is correct, then the rows represent a form of PSII that is not directly involved in linear electron transport, and could represent at least some of the 30% of PSII centers that are thought to be inactive [77].

In our opinion it is not clear yet if a structural organization of PSII in rows excludes a special long-range plastoquinone diffusion pathway between PSII in the rows and b_6/f in another part of the membrane. For instance, the organization in rows may permit a special route in the LHCII areas of the rows along which plastoquinone can migrate quickly. In Ref. [79] it was suggested that most of the lipids in the grana will be relatively immobile ‘boundary’ lipids. However, the number of boundary lipids can be significantly smaller than suggested in Ref. [79], because they will probably not be present in the regions between the long sides of the supercomplexes (as in the megacomplexes), while for a $C_2S_2M_2$ repeating unit the number of free LHCII trimers and thus the number of boundary lipids is also lower than calculated in Ref. [79]. Lower numbers of relatively immobile boundary lipids implicate higher numbers of highly mobile lipids and therefore plastoquinone diffusion may not be as restricted as calculated. This suggests that functional photosynthesis in semi-crystalline parts of the grana cannot be excluded yet.

4.5. Energy transfer and trapping in grana membranes

Efficient photosynthetic performance of PSII in grana requires a delicate interplay between excitation energy transfer, trapping of excitation energy by charge separation in the reaction center and subsequent secondary electron transfer reactions [156]. It is of interest to know to which extent the structural organization of PSII in the grana determines the kinetics and yield of these processes, and thus determines the photosynthetic performance of PSII.

There are in principle three different processes which could provide the rate-limiting step of energy transfer and trapping, i.e., the rate of the primary charge separation reaction, the rate of the energy transfer from the core antenna to the reaction center, and the rate of energy transfer among the various peripheral and core antenna proteins. If the overall kinetics is determined by one of these processes, the kinetics is said to be trap-limited, transfer-to-the-trap-limited, or diffusion-limited, respectively. In intact PSII, the overall kinetics occurs in two phases, with a main phase of about 200 ps and a minor phase of about 500 ps [157], provided that the quinone acceptor Q_A is oxidized. This kinetics is considerably slower than that observed for intact PSI (see Section 2).

There is currently no consensus on which of the processes mentioned above is most important for PSII in grana. A recent study on PSII complexes of different sizes was interpreted to mean that energy transfer and trapping in PSII is basically trap-limited [158]. However, it was recently pointed out that there is a long distance between chlorophylls of the PsbB or PsbC core antenna proteins and those of the reaction center, which implies a quite slow energy transfer reaction, and which, at least in the PSII core complex itself, could limit the trapping process [159]. On the other hand, it was pointed out that the orientations of the chlorophylls of PsbB and PsbC are optimized for fast excitation energy transfer [160] and a comparison between the energy transfer and trapping dynamics in CP47-RC and RC preparations suggested that the energy transfer between CP47 and the RC does not limit the overall trapping of the excitation energy [161].

Energy transfer within the peripheral antenna may in fact also be rate-limiting, because energy transfer from one LHCII to the next was suggested to occur in 32 ps [162] and, depending on the supramolecular organization, more than one LHCII complex needs to be passed before the excitation reaches the reaction center. Also the time of energy transfer between two opposing membranes in a granum is considerable [163,164]. We conclude that there are currently no data available that favor either a trap-limited or a diffusion-limited trapping of excitation energy in PSII *in vivo* (see also Ref. [84]). This implies that structural rearrangements of the peripheral antenna (which should primarily affect the diffusion-limited trapping rate) are likely to have effects on the photosynthetic performance *in vivo* (see also Section 6).

5. Overall chloroplast membrane topology

5.1. Vertical distance between membranes in grana stacks

The grana discs of green plant chloroplasts have diameters of about 300–600 nm [165,166]. The sizes of the grana and the proportions of grana and stroma appear remarkably constant among plant species and among plants grown under different environmental conditions [167].

In the vertical direction several to several dozens of membranes may stack. The membranes are never equidistantly spaced, because on the stromal side membranes are rather flat and seemingly form a pair spaced by about 2 nm, while at the luminal side the distance between the membranes is much larger because of the presence of large protrusions of the PSII core and cytochrome b_6/f complexes (see also Fig. 9). The vertical distance from one pair of membranes to the next can be extracted from micrographs of thin-sectioned chloroplasts. Table 2 gives a list of the average vertical repeat in the grana stacks, presented in or calculated from 17 different papers. Some of these numbers may give slight underestimations of the spacing, because the pressure used in the thin-sectioning technique to obtain slices of chloroplasts may induce some shrinking. Table 2 shows that the vertical repeat varies roughly from 14 to 24 nm, though distances around 16 and 21 nm seem to be the most common. A repeating unit of 21 nm would be just sufficient for the vertical height of two PSII or cytochrome b_6/f complexes on top of each other, because each PSII and b_6/f unit measures maximally about 10.5 nm [10,11,113]. This would mean that not only contacts between PSII and LHCII at their stromal sides determine the morphology of the grana, but also contacts between PSII and/or cytochrome b_6/f units in opposing membranes in the lumen. This is in line with studies on inside-out vesicles with exposed luminal side [168], which revealed considerable interactions between the luminal-exposed sides of the membranes. The most exposed proteins are the extrinsic PsbP and PsbQ proteins of PSII, so these proteins may not only function to optimize the inorganic cofactor requirement for water oxidation [119], but also may contribute to the shape of the grana stacks. A shorter distance than 21 nm would mean, if not artefactual, that two PSII units cannot be located with their extrinsic subunits directly opposed to each other in the lumen. This could diminish the diffusion of PSII complexes in the grana stacks and also limit the available volume for luminal proteins (see also below).

Interesting observations were made by Murakami and Packer [169], who reported a considerable shortening of the vertical distance by light, and by Albertsson [168], who showed an increased interaction between the luminal-exposed sides of the membrane by the light-induced acidification of the lumen. This could mean that the repeating distances of about 16 and 21 nm reflect grana in light- and dark-adapted conditions, respectively, and that

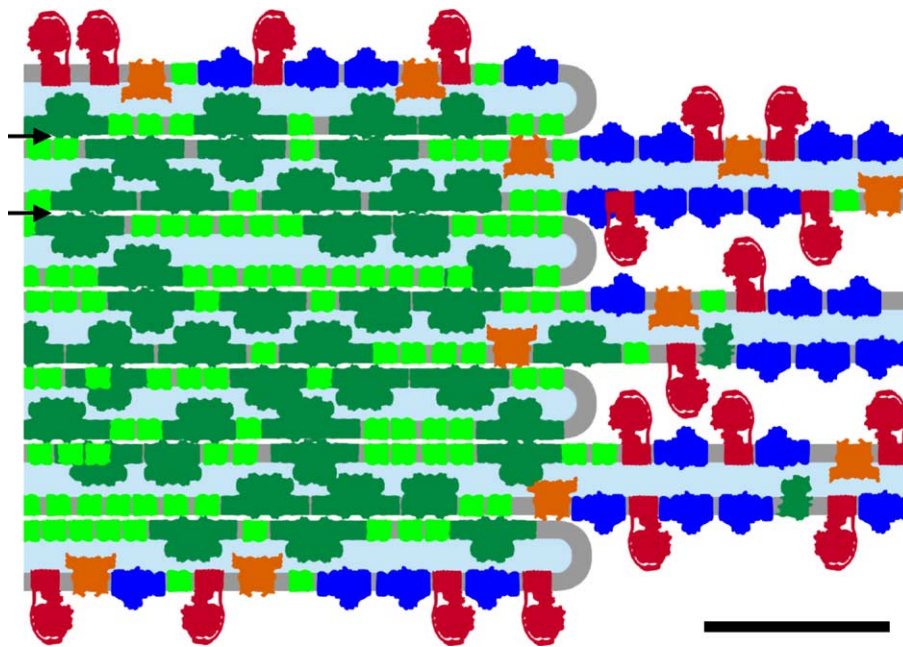


Fig. 9. A model of the green plant thylakoid membrane with dimensions derived from atomic models of the main protein components and cellular dimensions from thin sectioning electron microscopy. Dimeric PSII–LHCII supercomplexes and PSII monomers are in dark green, single LHCII trimers in bright green, PSI in dark blue, cytochrome b_6/f dimers in orange and ATP synthase in red. The acidic lumen is indicated in bright blue. The model shows about a half of an average grana stack in the horizontal dimension. PSII–LHCII supercomplexes can oppose both LHCII or other supercomplexes in opposing membranes. Note that under many physiological conditions, the number of cytochrome b_6/f dimers in the grana can be significantly higher than shown in this figure. The arrows indicate the vertical repeat as discussed in Section 5.1. The bar is 50 nm.

light induces a shrinking of the volume of the lumen in the stacks of up to 30% [170].

We note that the distance between the two membranes in the non-stacked regions of the chloroplasts can be considerably larger than in the grana (see, e.g., Ref. [166]), and that the protein complexes in the grana have considerably larger lumenal-exposed parts than those in the stroma, which suggests that most of the water-soluble proteins located in the lumen [171] will be present in the non-

appressed parts. In fact, the close spacing in the lumen between the opposing membranes in the stacks and the bulky extrinsic parts of PSII and/or cytochrome b_6/f may hinder the diffusion of water-soluble proteins in the lumenal compartments of the stacks.

5.2. Membrane curvature and margins

The question why the stacks appear flat is intriguing (they are already flat before LHCII is associated to PSII in the latest state of chloroplast development). Stroma membranes may be somewhat tubular, which could have to do with the F1F0 ATPase. The F1 headpiece is much larger than the membrane-bound F0 part, so two molecules would give steric hindrance if close together.

Another intriguing aspect of the morphology of the stacked membranes is the extreme curvature of the membranes in the margins, the part of the membranes that connect two grana membranes at their lumenal sides. A figure with all thylakoid components drawn on scale (Fig. 9) makes clear that at least the PSII, PSI and cytochrome b_6/f complexes are too large to be located in the margins. Of all thylakoid membrane complexes, the ATPase complex has the smallest diameter in the plane of the membrane, but there is no experimental evidence that this complex could be located in the margins. One should also realize that a protein complex that usually is located in a flat membrane will find it difficult to locate itself in a very strongly curved membrane. Based on these considerations, we conclude

Table 2
Vertical distance between pairs of membranes in grana stacks

Distance (Å)	Material	Reference
144	Spinach, Light adapted	[169]
147	Maize	[222]
152	Spinach, Av. 2 figures	[223]
155	Spinach, Av. 2 figures	[224]
162	Barley	[225]
165		[226]
173	Spinach	[227]
179		[228]
180	Spinach	[229]
189		[230]
195	Spinach	[231]
200		[13]
206	Av. 2 figures	[166]
214	Spinach, Dark adapted	[169]
215	<i>Arabidopsis</i> , Av. 2 figures	[232]
223	Barley, transversely fractured	[225]
230	Spinach	[233]
243	Spinach	[234]

that the strongly curved margins of the stacked membranes are essentially protein-free, as suggested earlier by Murphy [172].

The idea that the grana margins are protein-free seems to be in line with considerations of Staehelin and van der Staay [13], but contradicts quantitative models based on press treatment of thylakoid membranes followed by aqueous polymer two-phase partition (see, e.g., Refs. [173,174]). In these models, considerable numbers of at least three of the four types of large complexes are placed in the margins, which is inconsistent with the small volume of the strongly curved parts of the membranes. Based on the dimensions of grana and stroma, we calculate that the volume of the margins is at most 10% of the volume of the membranes in the grana, and at most 5% of the total volume of the thylakoid membranes. However, if the vesicles obtained by press treatment are just enriched in the parts of the membrane that originate from the margins before the press treatment, then the margins in the papers of Albertsson and co-workers also include considerable parts of the membranes adjacent to the strongly curved parts of the membranes and thus reflect the protein composition close to these curved parts.

5.3. Protein composition of grana and stroma

The PSI and ATP synthase complexes cannot be present in the grana membranes because of their bulky stromal-exposed parts and also not in the margins because of the large volumes of their membrane-intrinsic parts. So these complexes can only be located in unstacked thylakoid membranes and in the end membranes of the stacks (Fig. 9). PSII supercomplexes are probably located exclusively in the stacked grana membranes, while monomeric PSII core complexes can also occur in the stroma (see, e.g., Ref. [96]). The occurrence of monomeric PSII in the stroma thylakoids has physiological significance in view of the PSII repair cycle. Of all thylakoid proteins, the D1 protein of PSII is the most vulnerable for damage by light-induced radicals and active oxygen species, and all green plants have an efficient and rapid repair mechanism for damaged D1 [97]. This repair mechanism occurs in the stroma membranes, and thus requires a movement of a PSII supercomplex with damaged D1 protein from the grana to the stroma, a partial or complete disassembly of the complex into its individual subunits, proteolysis of the damaged D1 protein, insertion of nascent D1 chains in the membrane, followed by movement to the grana and assembly of the PSII–LHCII supercomplex. LHCII occurs for a large part in the grana, but to some extent also in the stroma, where it may bind to PSI (especially in the so-called State 2—see Section 6.1).

The location of PSII and PSI in physically separated parts of the membranes imposes constraints on the electron transfer between these systems, because long distances may have to be bridged. In this respect, the location of the

cytochrome b_6/f complex is crucial, just as the possibility or impossibility of long-range electron transfer from PSII to b_6/f by plastoquinone and from b_6/f to PSI by plastocyanin. Currently, most models assume an about even distribution of b_6/f between the grana stacks and stroma membranes (see, e.g., Refs. [173,175]) and a restricted diffusion of plastoquinone [76–78,176], suggesting the impossibility of long-range electron transfer by plastoquinone, at least in the relatively protein-rich grana.

We would like to point out that the location of the cytochrome b_6/f complex is not as clear as has been suggested in most studies. A large part of the experimental evidence for the about even distribution of b_6/f between grana and stroma was provided by biochemical studies (see, e.g., Refs. [177]) and electron microscopy of immunolabeled thylakoids [178,179]. However, the biochemical studies are rather indirect, and are in most cases based on the b_6/f contents of vesicles prepared by press treatment of thylakoid membranes followed by aqueous polymer two-phase partition [177]. It is unclear to which extent these vesicles represent the *in vivo* situation (see Section 5.2). The structural studies on immunolabeled thylakoids revealed a fair amount of b_6/f antibodies in the grana of broken chloroplasts, but in intact chloroplasts it seems that b_6/f in the grana occurs for a large part in clusters [178], whereas in another study a considerable amount of b_6/f antibody attributed to the grana may in fact arise from the end membranes [179], which certainly will have a different protein composition than membranes from the inner parts of the grana.

There are also indications that grana can be essentially depleted of the cytochrome b_6/f complex. Grana membranes isolated by Triton-X-100 treatment of stacked chloroplast membranes [180] were shown to consist of paired grana membranes with similar diameters as in native thylakoids [181], but do not contain any cytochrome b_6/f . A shorter and milder method using α -dodecylmaltoside also resulted in paired grana membranes which, however, also did not contain appreciable amounts of cytochrome b_6/f [117]. In the latter membranes, regular arrays of PSII and LHCII were frequently observed [149] with almost identical unit cells as found in untreated thylakoid membranes (see, e.g., Ref. [147] and Section 4.1), which suggests that under some conditions areas of PSII and LHCII can occur in grana membranes without significant numbers of cytochrome b_6/f complexes (see also Section 4.4). In the next section we propose an explanation for the presence or absence of the cytochrome b_6/f complex in the various grana preparations.

5.4. Factors that sustain the grana and stroma division

There are three main driving forces for the appearance of stacked and unstacked membranes, i.e., the interplay between van der Waals attractive forces and cation-mediated electrostatic interaction between membranes, lateral segregation of protein complexes within the membranes, and

steric hindrance. The final shape of the grana also depends on the type and percentages of lipids, and on the subtle interplay between the protein complexes and the lipids.

LHCII is generally considered to be the main protein responsible for the electrostatic interaction. Chl *b*-deficient chloroplast mutants are unable to produce normal amounts of membrane stacks [182]. The van der Waals attractive forces [183] and the cation-mediated electrostatic interaction between proteins in opposing membranes, in particular LHCII [184], are undisputed. Lateral segregation of protein complexes is also likely to be an important factor [185]. Lateral segregation of membrane proteins was demonstrated by reconstitution of an about 1:1 mixture of monodisperse cyanobacterial PSI and ATP synthase complexes into artificial phospholipid membranes after detergent removal [186]. In this system, the PSI monomers formed a lattice and have driven the ATP synthase complexes to the periphery of the vesicle (Fig. 10). Due to the low lipid to protein ratio, the vesicle is not continuous. Investigations showed that upon removal of ATP synthase from the mixture, the size of the crystals did not increase substantially, indicating a complex interplay of many factors such as the lipid composition in the ultimate size of these artificial crystalline membranes. The ability of PSII and LHCII to form various types of megacomplexes and (semi-)crystalline lattices is remarkable, just as the incapability of the ATP synthase complex to form two-dimensional close-packings or crystalline arrays (Fig. 10).

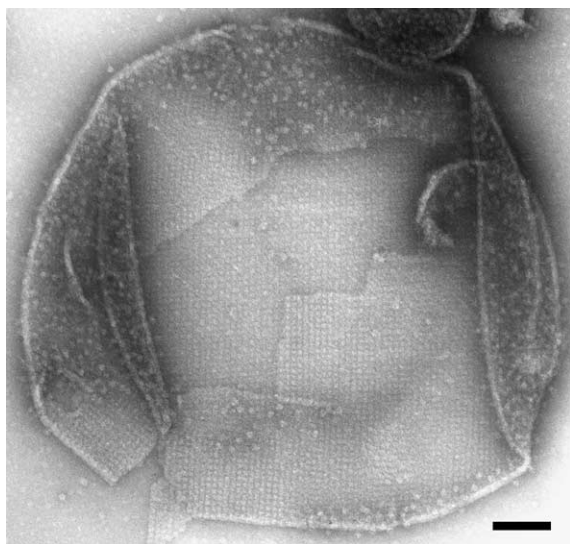


Fig. 10. Lateral segregation of membrane proteins, as demonstrated by reconstitution of an about 1:1 mixture of monodisperse cyanobacterial PSI and ATP synthase into artificial phospholipid membranes after detergent removal [186]. The lattice formed by the PSI monomers has driven ATP synthase complexes to the periphery. Due to the low lipid to protein ratio and air-drying during EM preparation the vesicle is not continuous. Investigations showed that upon removal of ATP synthase from the mixture, the size of the crystals did not increase substantially. This suggests a complex interplay of many factors such as the lipid composition in the ultimate size of these artificial crystalline membranes. The bar is 100 nm.

Steric hindrance prevents PSI and ATP synthase to move into stacked PSII enriched membranes, because of their very bulky stromal regions. For cytochrome *b₆/f* it was always assumed that there is no steric restriction to it being present in grana stacks (see, e.g., Refs. [175,187,188]). However, the new *b₆/f* structures reveal that subunit IV contains an extrinsic loop that may protrude slightly further into the space between the stacks than LHCII and PSII [10,11]. This could suggest that under severe stacking conditions the *b₆/f* complex is driven away from the most extensively stacked areas, leading to clusters of *b₆/f* complexes [178] or even to *b₆/f*-free areas of the grana membranes, like the crystalline domains of PSII–LHCII supercomplexes. Perhaps the *b₆/f* complex is ‘tolerated’ (or not pushed out) in the grana under normal physiological conditions, but excluded under conditions that lead to an increased stacking of the grana membranes.

5.5. Functional significance of stacking

One of the main consequences of stacking is the physical separation of PSI and PSII. It has been argued [189] that the separation of the antenna systems of PSI and PSII is essential for efficient photosynthesis, because the kinetics of the trapping of excitation energy is much faster in PSI than in PSII, and a location of both antenna system at short distances would lead to an uncontrolled flow of energy from PSII to PSI. The stacking not only prevents this spillover of excitation energy, but it also provides the chloroplast the means to fine-regulate the light need for photosynthesis [190] (see Section 6). The stacking also provides PSII a very large functional antenna, in which excitation energy can flow within a thylakoid membrane and between two stacked membranes until an ‘open’ PSII reaction center is found. In addition, it provides an easy means to adapt to low-light conditions, in which both the amount of LHCII and the extent of stacking have been shown to increase [191].

It is also possible that a physical separation of PSII and PSI is required to fine-tune the balance between linear and cyclic electron transport (see, e.g., Ref. [80] and references therein). In the linear scheme, electrons from water are transferred by way of PSII and PSI to NADP⁺. The cytochrome *b₆/f* complex mediates the electron transfer between the two photosystems, and generates a proton gradient which contributes to the formation of ATP. In the cyclic mode electrons generated on the acceptor side of PSI are transferred back, by way of the cytochrome *b₆/f* complex, to the donor side of PSI, which thus generate ATP without accumulating reducing equivalents. It has been shown that photosynthesis in green plants requires both electron transport pathways [192], and that the cyclic pathway operates very efficiently at the onset of illumination [193], which only can occur if the linear and cyclic electron transfer chains are physically separated from each other. Two different models have been proposed to explain these findings [80]. In the first, the cyclic pathway is

thought to take place in supercomplexes consisting of PSI, b_6/f , plastocyanin and ferredoxin. A problem of this hypothesis is that there is as yet no experimental evidence for the existence of such supercomplexes. In the second model, no such supercomplex is required, but a localization of both pathways in different parts of the membrane [80]. The linear pathway was suggested to occur preferentially near the grana margins, making use of PSII and b_6/f from the grana and nearby PSI complexes. The cyclic pathway would operate predominantly in stroma lamellae far away from the grana, with b_6/f located in the stroma. Ferredoxin-NADP reductase (FNR) would play a key role in discriminating between the linear and cyclic routes, by binding to PSI for linear electron transport and to b_6/f for cyclic electron transport. In the stacks, the binding of FNR to b_6/f is unlikely because of steric hindrance. We conclude that the occurrence of PSI and PSII in stroma and grana, respectively, and the more even distribution of the cytochrome b_6/f complex facilitate the balance between linear and cyclic electron transport.

6. Structural rearrangements of protein complexes upon short-term adaptation

6.1. State transitions

When plants are exposed to light conditions that preferably excite either PSI or PSII, then the plants are able to redistribute the excitation energy by a mechanism called ‘state transitions’ (see Refs. [74,175,194,195] for recent reviews). There is general consensus now about the basic features of this process. When PSII is preferentially excited by ‘light 2’, the plastoquinone pool becomes more reduced, which leads to a conformational change of the cytochrome b_6/f complex, which in turn activates a kinase bound to the b_6/f complex. This kinase is then released from b_6/f , after which it migrates to LHCII and promotes its phosphorylation. Phosphorylated LHCII is thought to have a decreased affinity for PSII and an increased affinity for PSI, and thus a lateral movement of phosphorylated LHCII from PSII to PSI occurs that can explain the shift from ‘state 1’ (induced by ‘light 1’) to ‘state 2’ (induced by ‘light 2’).

There are several aspects of this mechanism that are relevant for the understanding of the supramolecular organization of the protein complexes in the thylakoid membranes. The first relates to the identity of the kinases that catalyze the phosphorylation. A number of different types of kinases have now been described in *Arabidopsis* and *Chlamydomonas*, called TAKs (thylakoid-associated kinases) [196,197] and Stt7 (state transition thylakoid) [73]. Although these proteins are not sequence-related, their overall structures are comparable, with most probably a single transmembrane α -helix and a large extrinsic protein loop at the stromal side of the membrane. The size of this loop will most likely prevent the kinase to enter the region

between the stacks (Fig. 9), so only those LHCII complexes can be phosphorylated that are either in the stroma or at the interface between stroma and grana. This may explain why in green plants only a relatively small part of LHCII is phosphorylated, and perhaps also why high light intensities further limit the accessibility of LHCII for the kinase [198], because the observed decrease of the interthylakoid distance upon illumination may further limit the accessibility of LHCII (see Section 5.1).

It is not known if the LHCII that gets phosphorylated originates from the PSII–LHCII supercomplexes or from the LHCII-only part of the membranes. If phosphorylation only occurs at the border between grana and stroma, then it is possible that LHCII from both pools is actually involved. It has been reported that phosphorylation induces a dissociation of the LHCII trimer into monomers and a conformational change at the stromal side of the complex [199] but both statements require further experimental proof.

It is now clear which PSI subunits play an essential role in the state transitions. *Arabidopsis* plants without the PSI-H and PSI-L subunits [200] as well as those without the PSI-O subunit [201] are highly deficient in state transitions. The absence of these proteins did not have an effect on the phosphorylation of LHCII, and evidence was presented that phosphorylated LHCII remained bound to the grana in the absence of PSI-H [200]. Similar results were obtained with a PSI-deficient mutant from *Chlamydomonas* [202]. This means that the ‘molecular recognition’ between LHCII and the PSI-H subunit of PSI forms the molecular basis for the transition from state 1 to state 2 [175]. Biochemical studies have shown that both phosphorylated and non-phosphorylated LHCII bind to PSI [203], which suggests that neither phosphorylation nor the monomeric or trimeric aggregation state of LHCII affects the affinity of PSI for LHCII, and that phosphorylation will primarily affect the affinity of LHCII for the stacked grana membranes.

We note that, at least in *Chlamydomonas*, the transition from state 1 to state 2 is not only accompanied by the migration of LHCII from the grana to the stroma, but also by a migration of the cytochrome b_6/f complex [179] and by an increase of the extent of cyclic electron transport around PSI [204]. We also note that the extent of the state transitions is much larger in *Chlamydomonas* than in green plants: in green plants about 10–20% of LHCII is transferred from PSII to PSI upon the transition from state 1 to state 2 [205], whereas in *Chlamydomonas* about 80% of LHCII migrates from PSII to PSI [202]. These differences may be explained by differences in membrane structure in both types of organisms. *Chlamydomonas* membranes show a much less pronounced stacking than green plant membranes [206,207], which probably results in a much greater accessibility of LHCII for the kinases responsible for the phosphorylation of LHCII. This, in turn, could lead to a much larger proportion of LHCII that is able to migrate to PSI. It is not likely that the transition from state 1 to state 2 leads to a complete destacking of the thylakoid membranes

of *Chlamydomonas* [179], but involvement of the complete granum in *Chlamydomonas* instead of only a small part in green plants is likely. In this respect, it is interesting to note that PSII–LHCII super- and megacomplexes could only be isolated from *Chlamydomonas* membranes grown in state 1, but not in state 2 (A.E. Yakushevskaya, unpublished observations), suggesting that the association of the PSII and LHCII complexes is less tight in state 2 than in state 1. It is unlikely that the two types of PSI–LHCI particles found in *Chlamydomonas* (see Ref. [48] and Section 2.3) are related to the state transitions, because the size of the additional mass in the larger complexes is too small to explain the additional light-harvesting ability of PSI in state 2.

6.2. High-energy quenching

In most habitats, plants are exposed to a wide variety of irradiance intensities. Problems occur when the light intensity exceeds the plant's capacity for photosynthesis. Without proper protection, the accumulation of excited states will finally result in the generation of harmful oxygen species, which can damage the membranes, pigments and proteins of the photosynthetic organism [208]. In high light conditions, plants are able to harmlessly dissipate excess excitation energy into heat by a process known as high-energy quenching (qE). This process is one of the manifestations of the nonphotochemical quenching of chlorophyll fluorescence, in which the rate of non-radiative decay is increased, resulting in lower quantum yields of fluorescence, intersystem crossing to the triplet state and charge separation in the photosynthetic reaction centers. The basic features of this process are now well understood [209,210]. The process is triggered by acidification of the thylakoid lumen, which activates violaxanthin de-epoxidase (VDE), the enzyme that converts violaxanthin into zeaxanthin [211]. The process requires the presence of the PsbS protein [108] as well as its protonation by the acidification of the thylakoid lumen [212]. It has been shown that isolated PsbS is able to bind zeaxanthin, giving rise to spectroscopic changes that are also observed for qE in vivo [213]. Whether the activated zeaxanthin directly deactivates chlorophyll excited states (by means of energy transfer to the low-lying 'forbidden' first electronically excited S_1 state) or induces conformational changes in the (super) complexes of the grana (leading to new pigment–pigment interactions and increased non-radiative decay rates) is still a matter of debate [210].

The first aspect of this process relevant for the supramolecular organization of the photosynthetic apparatus is the location of the violaxanthin de-epoxidase enzyme. This enzyme is water-soluble and occurs in the thylakoid lumen. It was suggested that this enzyme requires lipid inverted hexagonal structures for its activity [214]. However, it was also shown that the inverted hexagonal phase of the major thylakoid lipid monogalactosyldiacylglycerol (MGDG) disappears upon the addition of LHCII [215], and so it must be

questioned to which extent VDE can catalyze the conversion of violaxanthin into zeaxanthin in a highly packed membrane like the thylakoid membrane of the grana (Fig. 9). There is more space for inverted hexagonal structures in the stroma lamellae, but in this case the zeaxanthin has to diffuse over a considerable distance to reach PsbS, because this protein is thought to be located exclusively in the grana. Perhaps the possibility could be investigated that VDE catalyzes the formation of zeaxanthin in the strongly curved margins of the thylakoid membranes. If VDE is active at the inner surface of these membranes, zeaxanthin would need to move over a relatively short distance to the PsbS protein in the grana membranes.

Another aspect relevant for the understanding of the supramolecular organization is the location of the PsbS protein. It was found that PsbS is not bound to PSII–LHCII supercomplexes [111] and also that it does not occur in crystalline arrays of $C_2S_2M_2$ supercomplexes in *Arabidopsis* [150], which excludes the possibility that the protein was lost from the PSII–LHCII supercomplexes during the purification procedure. This suggests that the PsbS protein is located in the LHCII-only regions of the grana membranes. Chlorophylls bound to proteins in these regions are presumably well able to transfer excitation energy to PSII (see Section 5.5), and a diminishing of excited state lifetimes in these areas of the membranes after zeaxanthin activation will give a quenching of fluorescence and the lower probability of exciting PSII. We note that part of the current controversy on the location of PsbS may be caused by the tendency of the isolated protein for self-aggregation [106]. A dimer-to-monomer conversion has been proposed upon acidification [109], but to which respect dimer formation at higher pH values is due to artificial aggregation is not clear. The finding that PsbS changes its conformation upon binding of zeaxanthin [213] is better documented, and a move of the activated protein from LHCII-only regions to PSII–LHCII supercomplexes remains a possibility.

7. Outlook

We have tried to give an answer to the question how the proteins in the thylakoid membranes are organized and how these membranes form the grana and stroma in the chloroplast. The classical electron microscopy techniques of freeze-etching, freeze-fracturing and negative staining have contributed a lot to the understanding, together with biochemical approaches. However, they have some limitations because the chloroplast is disrupted before imaging. Thus, there always remains some bias about the credibility of the observed positions of the membranes and their individual components. An alternative would be to perform tomographic reconstructions of intact chloroplasts just as was carried out for mitochondria [216]. It is expected that the resolution will increase to 2–3 nm in the near future by improving data acquisition techniques and the use of

helium-cooled electron microscopes. With the present state-of-the-art tomography, it should for instance be possible to visualize the membrane system of stacked and unstacked thylakoid membranes. Application of cryo-EM at liquid helium temperatures would also be advantageous for single-particle averaging.

Another promising technique that was, as yet, not much applied to the study of the organization of green plant thylakoid membranes is AFM, with which a number of spectacular results were obtained on membranes and two-dimensional crystals of photosynthetic complexes from purple bacteria [217–219]. With this technique topographs can be obtained of membranes at a higher signal-to-noise ratio than with electron microscopy. Also the combination of this technique with two-photon fluorescence imaging seems promising [220]. For oxygenic photosynthetic systems studies on two-dimensional crystals of cyanobacterial PSI [221] and on envelope-free chloroplasts [165] were presented. The latter study is particularly interesting, because it allowed a first insight into rearrangements of the photosynthetic membranes upon unstacking.

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